Vol. XV. No. 2. JUNE, 1961

THE BRITISH JOURNAL OF CANCER

(The official journal of the British Empire Cancer Campaign)

EDITOR
R. W. Scarff

ASSISTANT EDITOR
A. C. Thackray

ADVISORY EDITORS
Sir Samuel P. Bedson
Sir Stanford Cade
Sir Charles Dodds
Malcolm Donaldson
C. E. Dukes
L. H. Gray
A. Haddow
W. D. Newcomb
Sir Heneage Ogilvie
Percy Stocks
Sir Brian Windeyer

Copyright © 1961
LONDON
H. K. LEWIS & CO. LTD.
136 GOWER STREET, W.C.1

Published Quarterly
Annual Subscription, post free, Four Guineas
Single copies, One Pound Five Shillings net

EDITORIAL.

Scope of the Journal.—For the publication of original communications concerning the causes, distribution, symptoms, pathology and treatment of malignant diseases and allied conditions. Papers upon clinical, bacteriological, biochemical, pharmacological, physiological, radiological, serological, and other subjects therefore would be acceptable when related to this subject.

Forwarding Papers and Correspondence.—Papers should be forwarded to Professor R. W. Scarff, The Middlesex Hospital, London, W.1.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C.1.

Date of Publication.—The Journal is published quarterly in March, June, September and December.

Rapidity of Publication.—All papers accepted for publication will be printed, so far as is possible, in the next following issue of the Journal, if received before the first day of the month preceding publication. Long papers, or papers with illustrations, may, however be delayed if not received earlier.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers before the issue is printed. Prices on application.

BRITISH JOURNAL OF CANCER

VOL. XV JUNE, 1961 NO. 2

GASTRO-INTESTINAL CANCER AND GEOCHEMISTRY IN NORTH MONTGOMERYSHIRE

IAN B. MILLAR

*Late Medical Officer of Health, North Montgomeryshire, Borough Council Offices, Welshpool**

Received for publication February 18, 1961

THIS study commenced in 1956 as an enquiry into cancer mortality in the Rural District of Llanfyllin and in due course it was extended to include the other four districts of North Montgomeryshire. In 1921–30 Montgomery was one of the four central counties of Wales shown by Stocks to have a moderate excess of stomach cancer over that for England and Wales (Stocks, 1947). The five northern counties had the greatest excess however with their age and sex group death-rates varying from 146 to 203 per cent of the corresponding national rates. In 1947–54 the standardised mortality from stomach cancer, taking the national figure at 100, exceeded 150 in 26 out of 54 administrative areas of North Wales, varying from 200 to 260 in nine of these, but it exceeded 150 in only two out of 33 areas in Cheshire and Lancashire (Stocks, 1958). Did mortalities of this order extend to the central counties of Wales during the same period, in particular to North Montgomeryshire? If so, would it be possible to elucidate the aetiology of gastric cancer in any way by attempting to find factors associated with this increased mortality, especially factors of an environmental nature? The standardised mortalities of the four areas immediately adjacent to North Montgomeryshire were 160, 236, 170 and 123 from west to east in the period 1947–54 and the standardised mortalities for Montgomeryshire itself during the period 1950–53 were 138 for males and 143 for females (Registrar-General, 1951). The Rural District of Llanfyllin was chosen for the first part of the study for the following reasons :

1. It had the largest population of the five authorities of North Montgomeryshire.

2. Its northern border and those of the four areas of high mortality already mentioned were practically conterminous.

3. It was " truly rural " in nature (Registrar General, 1953) and its population was comparatively stable over a long period of time.

The average total cancer death-rate in Llanfyllin R.D. for the period 1933–53 was 1968 per million and in 1955 it was 2018 per million (unstandardised). In England and Wales in 1955 the rate was 2055 per million (male 2252, female1873). If comparison is confined to the age groups over 45 years of age, the number of deaths to be expected in Llanfyllin R.D. over the ten-year period 1946–55, based upon the rate of 5148 per million at ages 45 and over in England and Wales during

* Present address : 120 Wood Street, Chelmsford, Essex.

EDITORIAL.

Scope of the Journal.—For the publication of original communications concerning the causes, distribution, symptoms, pathology and treatment of malignant diseases and allied conditions. Papers upon clinical, bacteriological, biochemical, pharmacological, physiological, radiological, serological, and other subjects therefore would be acceptable when related to this subject.

Forwarding Papers and Correspondence.—Papers should be forwarded to Professor R. W. Scarff, The Middlesex Hospital, London, W.1.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C.1.

Date of Publication.—The Journal is published quarterly in March, June, September and December.

Rapidity of Publication.—All papers accepted for publication will be printed, so far as is possible, in the next following issue of the Journal, if received before the first day of the month preceding publication. Long papers, or papers with illustrations, may, however be delayed if not received earlier.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers before the issue is printed. Prices on application.

BRITISH JOURNAL OF CANCER

VOL. XV JUNE, 1961 NO. 2

GASTRO-INTESTINAL CANCER AND GEOCHEMISTRY IN NORTH MONTGOMERYSHIRE

IAN B. MILLAR

*Late Medical Officer of Health, North Montgomeryshire, Borough Council Offices, Welshpool**

Received for publication February 18, 1961

THIS study commenced in 1956 as an enquiry into cancer mortality in the Rural District of Llanfyllin and in due course it was extended to include the other four districts of North Montgomeryshire. In 1921–30 Montgomery was one of the four central counties of Wales shown by Stocks to have a moderate excess of stomach cancer over that for England and Wales (Stocks, 1947). The five northern counties had the greatest excess however with their age and sex group death-rates varying from 146 to 203 per cent of the corresponding national rates. In 1947–54 the standardised mortality from stomach cancer, taking the national figure at 100, exceeded 150 in 26 out of 54 administrative areas of North Wales, varying from 200 to 260 in nine of these, but it exceeded 150 in only two out of 33 areas in Cheshire and Lancashire (Stocks, 1958). Did mortalities of this order extend to the central counties of Wales during the same period, in particular to North Montgomeryshire? If so, would it be possible to elucidate the aetiology of gastric cancer in any way by attempting to find factors associated with this increased mortality, especially factors of an environmental nature? The standardised mortalities of the four areas immediately adjacent to North Montgomeryshire were 160, 236, 170 and 123 from west to east in the period 1947–54 and the standardised mortalities for Montgomeryshire itself during the period 1950–53 were 138 for males and 143 for females (Registrar-General, 1951). The Rural District of Llanfyllin was chosen for the first part of the study for the following reasons :

1. It had the largest population of the five authorities of North Montgomeryshire.

2. Its northern border and those of the four areas of high mortality already mentioned were practically conterminous.

3. It was " truly rural " in nature (Registrar General, 1953) and its population was comparatively stable over a long period of time.

The average total cancer death-rate in Llanfyllin R.D. for the period 1933–53 was 1968 per million and in 1955 it was 2018 per million (unstandardised). In England and Wales in 1955 the rate was 2055 per million (male 2252, female 1873). If comparison is confined to the age groups over 45 years of age, the number of deaths to be expected in Llanfyllin R.D. over the ten-year period 1946–55, based upon the rate of 5148 per million at ages 45 and over in England and Wales during

* Present address : 120 Wood Street, Chelmsford, Essex.

the almost similar period 1946–57, would be 199·5. This figure is based upon the 1951 Census (General Registrar Office, 1951) population level and takes into account the increased number of old people in the district. The actual figure, which was 202, might have suggested a reasonably satisfactory state of affairs until an analysis according to site revealed that the higher national rates for cancer of the lung in the male and genital cancer in the female were balanced by the excess of cancer of the digestive system in the Welsh district (Table I).

TABLE I.—*Cancer Death-rates According to Site for All Ages During the Period 1946–59 in Llanfyllin R.D. and 1946–57 in England and Wales*

International statistical classification number	Site	Rates per million				Proportion per 1000 total cancer deaths			
		Males		Females		Males		Females	
		L.	E. and W.	L.	E. and W.	L.	E. and W.	L.	E. and W.
141	Tongue	—	—	15	—	—	—	7	—
143–148	Buccal cavity and pharynx	28	—	72	—	12	—	37	—
140–148	—	—	73	—	27	—	34	—	15
150	Oesophagus	149	68	102	38	70	32	51	21
151	Stomach	597	382	450	275	275	178	228	149
152–153	Intestine	353	209	377	254	163	98	192	138
154	Rectum	149	168	72	108	68	79	37	59
155–156	Biliary passages and liver	28	51	58	58	12	24	29	32
157	Pancreas	54	79	72	64	25	37	37	35
140–159	Total digestive system	1358	1030	1218	824	625	482	618	449
161	Larynx	13	36	44	8	7	17	22	5
162–163	Lung, bronchus, trachea, pleura	218	556	44	93	99	259	22	50
160–165	Total respiratory system	231	592	88	101	106	276	44	55
170	Breast	—	—	232	358	—	—	118	195
171–174	Uterus	—	—	131	179	—	—	65	97
175	Ovary	—	—	58	110	—	—	30	60
177	Prostate	231	149	—	—	107	69	—	—
170–179	Total genital organs	231	149	421	647	107	69	213	352
180	Kidney	—	29	15	18	—	14	7	10
181	Bladder	54	84	—	34	25	39	—	18
180–181	Total urinary system	54	113	15	52	25	53	7	28
190–191	Skin	41	24	29	20	18	11	15	11
193	Brain, nervous system	—	35	—	23	—	17	—	13
194	Thyroid	14	—	29	—	7	—	15	—
196	Bone	68	21	—	15	31	10	—	8
200	Lymphosarcoma and reticulosarcoma	—	19	—	12	—	9	—	7
201	Hodgkin's disease	40	23	29	12	18	11	15	6
204	Leukaemia and aleukaemia	68	49	72	39	31	23	37	21
195, 197 198, 199 (also 194 in England and Wales)	All other and unspecified	68	92	70	94	32	39	36	50
140–205	Total cancer	2173	2147	1971	1839	—	1000	—	—
	Total less male lung cancer and female genital cancer	1942	1555	1550	1192	—	—	—	—

The population of this district was too small to yield a sufficient annual number of cancer deaths for analysis so that it was necessary to magnify it artificially by

extending the period under review to nearly fourteen years. The years originally chosen were 1946–55 because they pivotted on the census year 1951, thereby gaining some stability to help to counteract the weakness inherent in an analysis of small numbers. Then the period was extended to November 1959 in order to lessen inaccuracy but in fact all the rates and calculations remained substantially the same. The 1951 census statistics helped greatly to dispose of the age and sex variations by showing what adjustments should be made.

If a population is subjected to the effects of a noxious agent and other factors are distributed at random, then it might be expected that the social classes would be affected in equal numbers. In England and Wales there was no marked variation of mortality with social class for cancer of all sites at the 1951 census. Conversely, a variation in social class mortality might suggest a specific cancer provocative or even a particular carcinogen. In cancer of the stomach there was a definite social gradient of rising mortality with lowering of social class although there was at the same time an opposite trend in regard to cancer of the intestine (Registrar-General, 1951). The death rate for intestinal cancer surpassed that for stomach cancer at the 65–74 age period and the average age at death was slightly higher for intestinal than for stomach cancer. Local statistics supported these trends (Tables II and X). Assuming an equal distribution of resistance to cancer over the social classes, other factors such as diet and cooking methods might have determined a disproportion in mortality in such a way that a lesser dosage of carcinogen might have had a delayed effect upon the intestine or there might have been a different pattern of selective absorption, in either case causing death at a later age and at the opposite end of the social scale among those susceptible to cancer but hitherto unaffected by it.

Cancer of the stomach

It will be seen from Table I that the local mortality from cancer of the stomach for both sexes is considerably in excess of the figures for England and Wales over a similar period. That this is not a feature peculiar to Llanfyllin R.D. is revealed if the rates for rural districts in Wales are expressed as percentages of the 1951 Equivalent Average Death Rates for stomach cancer at ages 35–74 in England and Wales. These were 165 per cent (male) and 174 per cent (female) (Registrar-General, 1954). When these increases have been applied to the rates in Table I for England and Wales, they become 630 (male) and 478 (female) per million which accord well with the actual rates for Llanfyllin R.D.

As the number of old people is known to be excessive in these rural districts, due account must be taken of any influence exerted by them on the death rates. The 1951 Census showed that there were one and a half per cent more people over the age of 65 years in Llanfyllin R.D. than in the country as a whole, and that this excess was attributable to males (Table IV). In the magnified population this would mean 2355 extra males over the age of 65 years. If these males are then allocated to the 65–74, 75–84 and the Over 85 age groups as at the 1951 Census, 69 per cent or 1625 would be placed in the first groups, 26 per cent or 612 in the second group and 5 per cent or 118 in the third group. Applying to these numbers the appropriate death rates (England and Wales, 1946–57) shown in Table III, there would be 5·4 deaths above the 28·1 to be expected on the national rate making a total of about 33 deaths against the actual number of 44. This gives

TABLE II.—*Cancer Deaths According to Age, Site and Social Class in Llanfyllin R.D. During* 1946–59

Site	Male			Female		
	Average age (years)	Number of deaths	Percentage in social classes I and II	Average age (years)	Number of deaths	Percentage in social classes I and II
Stomach	66·6	44	43·2	69·1	31	58·1
Oesophagus	62	11	45·5	68·1	7	71·4
Intestine	67·1	26	73·1	72·1	26	46·2
Rectum	66	11	36·4	72	5	40
Pharynx etc.	39·5	2	50	60	5	60
Tongue	—	—	—	72	1	(Class II)
Pancreas	75·2	4	75	65·6	5	60
Gall bladder	71	2	100	—	—	—
Liver	—	—	—	66·2	4	100
Larynx	56	1	(Class V)	66	3	33·3
Lung	58·9	16	56·3	60	3	66·6
Breast	—	—	—	63·4	16	50
Uterus	—	—	—	52·2	9	33·3
Ovary	—	—	—	56·5	4	75
Prostate	75·5	17	35·3	—	—	—
Kidney	66	1	(Class II)	71	1	(Class III)
Bladder	65·6	3	100	—	—	—
Skin	84·3	3	33·3	74	2	50
Thyroid	72	1	(Class II)	64	2	50
Bone	42·4	5	40	—	—	—
Leukaemia	44	5	40	52	5	40
Hodgkin's disease	54	3	33·3	53	2	Nil
Others	58·5	5	—	74·4	5	—
Totals	64·4	160	50	66·2	136	52·2

Note : The average age at death from all causes for both sexes during the same period was 69·2 years.

a mortality ratio of 133·3 which may be compared with the actual S.M.R. of 135 based upon the expected number of deaths as calculated by applying the specific death rates for England and Wales of Table III to the appropriate groups of the 1951 Census population for Llanfyllin R.D.

The effect of migration has been taken into account in these calculations and in the preparation of Table III. The population in 1956–59 was about 5 per cent less than that of the period 1946–55. Age groups under 65 years were therefore

TABLE III.—*Cancer of the Stomach : Sex and Age Specific Death Rates per Million in Llanfyllin R.D. During* 1946–59 *and in England and Wales During* 1946–57

		Age groups				
		45–54	55–64	65–74	75–84	85 and over
Male :	Llanfyllin R.D.	598	1859	1932	5133	—
	England and Wales	349	978	2036	2956	2550
Female :	Llanfyllin R.D.	202	755	2232	3761	2008
	England and Wales	160	450	1157	2169	2390
Deaths :	Male	6	14	12	12	Nil
	Female	2	6	13	9	1

dealt with according to the following formula in which P represents the number of persons in the group and 10 and 3·83 the number of years in the two periods. The magnified population was therefore $10\,P + 3{\cdot}83\left(P - \frac{P}{20}\right)$.

In the case of females, where practically no adjustment for population over 65 years is necessary, the expected number of deaths based upon the national rate is 19 against the actual figure of 31. This gives a mortality ratio of 163·16 which may be compared with the actual S.M.R. of 161·5 based upon the expected number of deaths as calculated by applying the specific death rates in the same way as for males. The various ratios of the gastric mortality rates for Llanfyllin R.D. to those for England and Wales may be summarised as follows :

	A	B	C
Female	163·6	163·16	161·5
Male	156·3	133·3	135
Excess female ratio	7·3	29·86	26·5

A : Ratios based upon total population.
B : Ratios based upon total population after adjustment for population aged 65 years and over.
C : Standardised mortality ratio.

The difference of 26·5 in the S.M.R. between the sexes in Llanfyllin R.D. is due largely to the greater proportion of older men in the community. In the absence of this excess of older men the S.M.R. might have been expected to approximate the figure given in column A. An environmental carcinogenic factor might be expected therefore to affect females only 7 per cent more often than males, which is virtually insignificant in view of the numbers involved.

It is evident that the excess of deaths is genuine, particularly as the proportion of old people in Llanfyllin R.D. is not much different from that in the neighbouring counties or in rural Wales. The increase in the percentage of those over 45 years since the 1931 Census was also much the same in these districts as in England and Wales (Table IV).

TABLE IV.—*Populations : Proportions Over 45 Years*

	Percentage over 45 years at the Census, 1951	Percentage over 45 years in 1955	Percentage increase between 1931 Census and the 1951 Census
Llanfyllin R.D.	37·6	—	—
Wales (Region II)	—	38·8	—
England and Wales	35·1	36·6	6·0 (male 5·1) (female 6·8)
Counties of Merioneth, Montgomery and Radnor	37·6	—	5·1 (male 3·26) (female 7·25)

Percentage of populations over 65 years at the 1951 Census

	Male	Female	Total
England and Wales	9·0	12·6	10·83
Llanfyllin R.D.	12·2	12·63	12·41
Forden R.D.	11·74	13·18	12·45
Welshpool M.B.	9·25	12·63	10·88
Llanfyllin M.B.	13·4	15·5	14·5
Montgomery M.B.	12·8	13·3	13·0

Note : Region II of Wales consists of all the non-industrial counties.

There has been a gradual increase in the proportion of old people in the population of England and Wales but, in addition, there has been a depopulation in Mid-Wales affecting Montgomeryshire in particular. This has resulted in a fairly steady trend downwards over the past 70–80 years. The exodus seems to have been initiated and maintained by the 15–24 age group, especially females, followed by lessening numbers of both sexes in the later age groups of the working period of life. This has become almost a stable trend in that the depopulation takes away potential family makers and therefore potential old people leaving an almost constant excess in this area of 2–3 per cent in the proportion aged 45 years and over.

There is also in Table III a suggestion of earlier death from gastric cancer in Llanfyllin R.D., and at least it can be said that the malevolent force is fully active in the younger age groups. The average age at death however, is below the average for all deaths in the district by about the same amount as was found in England and Wales generally in 1955.

When the deaths are spread over the occupational social classes according to the occupation recorded on the death certificate it is found that approximately half of the deaths occur in Class II (Tables II and V and Fig. 2). Table V shows that

TABLE V.—*Proportion per* 1000 *Occupied or Retired Males Aged* 15 *Years and Over in Social Classes*, 1951

	Social classes				
	I	II	III	IV	V
Llanfyllin R.D.	24	382	236	264	94
Montgomeryshire	25	329	318	227	101
Merionethshire, Montgomeryshire, Radnorshire	31	288	373	216	92
England and Wales	34	155	526	159	126
Proportion per 1000 deaths in the social classes in Llanfyllin R.D. in 1946–59 and England and Wales in 1950					
Llanfyllin R.D.—					
Total cancer	34 (10)	483 (141)	281 (82)	113 (33)	89 (26)
Stomach cancer (male)	—	442 (19)	256 (11)	209 (9)	93 (4)
(female)	—	600 (18)	166 (5)	100 (3)	134 (4)
(total)	—	507 (37)	219 (16)	165 (12)	109 (8)
Intestinal cancer (male)	77 (2)	654 (17)	115 (3)	154 (4)	—
(female)	115 (3)	345 (9)	345 (9)	41 (1)	154 (4)
(total)	96 (5)	500 (26)	231 (12)	96 (5)	77 (4)
England and Wales—					
Stomach cancer (male)	24	141	460	188	187
(female)	25	148	483	176	168
(total)	24	143	466	185	182

Note : The actual numbers of deaths in Llanfyllin R.D. are shown in brackets.

cancer deaths in Llanfyllin R.D. are disproportionately numerous in Classes I, II, and III, especially stomach cancer in Class II and intestinal cancer in Classes I and II. The table also shows that the proportions in Classes II and IV are greatly in excess of those for England and Wales. This is of course due to the numbers of farmers and agricultural workers in these groups. Some attention must therefore be paid to the mortality rates of these two sub-divisions of Classes II and IV.

This is not difficult in Llanfyllin R.D. as Classes II and IV are themselves almost identical with their sub-divisions (apart from one death in each sex). It implies a high rate among farmers though not among agricultural workers. The question then arises of the rate among farmers in the country as a whole and Table VI shows that the standardised mortality among farmers, although above that for their class, is decidedly below the average for the general population. The standardised mortality among agricultural workers is, if anything, below the average for their class. For all causes of death however, the male standardised mortality ratio is less among farmers than others in Class II, and the female mortality shows little difference, thus accentuating the relatively greater standardised mortality ratio for stomach cancer in the case of the male farmer.

TABLE VI.—*Cancer of Stomach: Standardised Mortality for Social Classes in England and Wales*, 1951.

	Social classes				
	I	II	III	IV	V
Males aged 20–64 years	57	67	100	114	132
Farmers	—	88	—	—	—
Agricultural workers	—	—	—	114	—
Females aged 20–64 years	57	72	101	106	138
Farmers	—	91	—	—	—
Agricultural workers	—	—	—	102	—
Stomach cancer death rates per 10,000 all causes					
Males over 65 years	206	254	298	325	333
Farmers	—	292	—	—	—
Agricultural workers	—	—	—	290	—
Others Class IV	—	—	—	345	—
Females over 65 years	257	280	327	336	341
Farmers	—	303	—	—	—
Agricultural workers	—	—	—	394	—
Others Class IV	—	—	—	304	—

Standardised mortality : all causes	Male			Female		
	20–64 years	65–70 years	Over 70 years	20–64 years	65–70 years	Over 70 years
Class II	86	91	97	84	91	94
Farmers	73	76	94	91	89	99
Class IV	94	92	100	104	114	104
Agricultural workers	80	76	104	102	139	111

Table VII shows an expected high percentage of farmers in Llanfyllin R.D. and it will be noted that the figure for all those engaged in farm work (54·9 per cent) tallies well with the corresponding figure for stomach cancer deaths (56·3 per cent). It would not appear that any special proclivity to this disease obtains among the farming community. The explanation of the disparity between the rates for farmers and agricultural workers might be that some of the latter had been classified as farmers on the death certificates. Many farm labourers also have a few acres of land attached to their places of residence which they work in the style of a small farmer.

TABLE VII.—*Percentage of Occupied Males as Farmers (A) and Agricultural Workers (B)*

	A	B	A + B
England and Wales	1·87	3·85	5·72
Wales	4·53	3·88	8·41
Denbighshire	6·72	6·8	13·52
Merionethshire	12·0	8·3	20·3
Montgomeryshire	23·2	17·1	40·3
Radnorshire	23·7	17·9	41·6
Machynlleth R.D.	30·0	20·4	50·4
Llanfyllin R.D.	31·7	23·2	54·9
Newtown and Llanidloes R.D.	34·5	21·9	56·4
Forden R.D.	33·8	23·4	57·2
Penllyn R.D. (Merionethshire)	41·0	24·3	65·3

Llanfyllin R.D. Farmers and Agricultural Workers

Percentage stomach cancer	(male)	37·2	11·6	48·8
	(female)	53·3	10·0	63·3
	(total)	45·0	11·3	56·3
Percentage intestinal cancer	(male)	53·8	11·5	65·3
	(female)	28·0	—	28·0
	(total)	40·4	5·7	46·1
Percentage lung cancer	(total)	31·6	10·5	42·1

Percentages of non-cancer deaths attributable to Farmers and Agricultural Workers, 1953–59

Llanfyllin R.D.	42·1	6·46	48·56
Forden R.D.	34·0	12·4	46·4
Welshpool M.B.	14·1	6·4	20·5
Montgomery M.B. (1954–59)	23·2	11·6	34·8
Llanfyllin M.B. (1954–59)	23·4	7·8	31·2

Cancer of the intestine

As in the case of stomach cancer, the local rates are in marked excess of those for England and Wales over a similar period (Table I). The estimated number of deaths as against the actual number has been calculated in the same way as for stomach cancer and the results are set out in Table VIII. The agreement with the standardised mortality ratio is again reasonably good, especially in regard to female deaths.

TABLE VIII.—*Relative Intestinal Cancer Mortalities*

	Male	Female
Expected deaths (based on England and Wales rates)	15·39	16·5
Expected deaths due to older age of the population in Llanfyllin R.D.	1·54	1·4
Total expected deaths	16·93	17·9
Actual deaths	26·0	26·0
Actual deaths as percentage of expected deaths	153·6	145·3
Standardised mortality ratio	142·9	149·8

Since the period 1931–39 there has been a steady decrease in intestinal cancer mortality in England and Wales until in 1950–54 it was 78·2 per cent (male) and 76·9 per cent (female) of its former level. The standardised rates for England and Wales had been building up to a peak in the years 1931–39, but even these rates were less than those prevailing in Llanfyllin R.D. It cannot be argued therefore that the high Llanfyllin rate is merely a local failure in the national mortality decline. It is more likely to have been above national levels at all times and to

have been thrown perhaps into greater relief recently with the declining mortality elsewhere.

The distribution of the sex and age specific death rates are shown in Table IX in which the heavier local death rate is seen to be reflected in nearly all the main age groups. The average age at death is a little higher in intestinal than in stomach cancer (Table X)

TABLE IX.—*Intestinal Cancer: Sex and Age Specific Death Rates per Million in Llanfyllin R.D. During* 1946–59 *and in England and Wales During* 1946–57

	Age groups					
	35–44	45–54	55–64	65–74	75–84	85 and over
Male: Llanfyllin R.D.	99	199	797	1610	2566	2331
England and Wales	44	130	392	1127	2273	2592
Female: Llanfyllin R.D.	—	202	377	1545	4596	2008
England and Wales	52	162	409	952	2064	2963
Actual numbers of deaths: Male	1	2	6	10	6	1
Female	—	2	3	9	11	1

TABLE X.—*Average Age at Death*

	England and Wales		Llanfyllin R.D.	
	Male	Female	Male	Female
	(years)	(years)	(years)	(years)
Stomach cancer	66·16	69·8	66·6	69·1
Intestinal cancer	68·8	70·0	67·1	72·1

Note: The national figures are based on statistics for the period 1950–55 obtained from Studies on Medical and Population Subjects, No. 13 (General Register Office), using the mid-1953 population, and the local figures apply to the years 1946–59.

The female 75–84 age group, which had the highest number of deaths has no obvious common factor. The deaths are distributed evenly throughout the period of time, geographically they are evenly distributed, and their proportions in the social classes are similar to those of the deaths as a whole.

As in the case of stomach cancer, Social Classes II and IV consist mainly of farmers and agricultural workers and Table XI shows again a tendency to higher standardised mortality ratios for these occupations, especially farmers. It also shows that intestinal cancer, unlike stomach cancer, has a tendency to affect Classes I and II more than IV and V. The same applies to Llanfyllin R.D. where there were nearly as many deaths due to intestinal as to stomach cancer in Classes I and II. Certainly, taking these two classes together, there is a greater proportion of intestinal than stomach cancer (Table V and Fig. 2).

The over-all percentage of cancer of the intestine in the male agricultural population is appreciably greater than the percentage that that population bears to the total occupied males (Table VII). The same explanation for the disparity between the rates for farmers and agricultural workers in regard to stomach cancer applies to this cause of death in that many agricultural labourers also have a few acres of ground and so may be classified as small farmers. The female rate attributable to farming occupations is not so convincing however and yet, knowing

TABLE XI.—*Intestinal Cancer: Standardised Mortality of the Social Classes in England and Wales*, 1951

	Social class				
	I	II	III	IV	V
Males aged 20–64 years	123	100	105	85	94
Farmers	—	117	—	—	—
Agricultural workers	—	—	—	90	—
Females aged 20–64 years	103	100	97	104	106
Farmers	—	113	—	—	—
Agricultural workers	—	—	—	100	—
Intestinal cancer death rates per 10,000 all causes					
Males aged 65 years and over	210	211	192	187	192
Farmers	—	214	—	—	—
Agricultural workers	—	—	—	198	—
Females aged 65 years and over	375	291	262	271	275
Farmers	—	254	—	—	—
Agricultural workers	—	—	—	277	—

the excessive mortality among females, it may well be understated. There were six among those classified under other occupations who could have had farming associations stronger than the mere fact of living in a farming community. This also may apply to stomach cancer deaths only not to the same extent.

The other districts

The study was extended to the other four districts of North Montgomeryshire covering the period 1953–59 for the Borough of Welshpool and Forden R.D. and 1954–59 for the Boroughs of Montgomery and Llanfyllin. Three of these districts contained small concentrations of old people sufficient to affect the death rates of each district. Taken together however, there was no appreciable effect of this kind because most of these old people originated within the five districts forming the area of this enquiry. The Borough of Llanfyllin has accommodation for old people (59 at the time of the census), and this is reflected in the high proportion of the population over the age of 65 years (Table IV). These 59 people represented about 0·5 per cent of the combined population of the Borough and Rural District of Llanfyllin, yet they contributed to cancer deaths and stomach cancer deaths to the extent of 3 and 4 per cent respectively. On the other hand Welshpool, though possessing a small home for old people (about 12), was the only district with a proportion of persons over 65 years similar to that in England and Wales. The social class representation is also similar to that in England and Wales (Fig. 1 and 4), both for population and cancer mortality. A hospital for mental defectives in the Forden R.D. (140 inmates at the time of the census) also has a department for the chronic sick of Forden and the other four districts. Less than twenty beds were being used for this purpose during part of the time covered by this study.

The comparative rates for gastro-intestinal cancer in these three districts are shown in Table XII. When compared with the rates for Llanfyllin R.D. shown in Table I, it will be seen that, despite the concentration of old people in the other districts, Llanfyllin R.D. has virtually the highest rates. The more strictly comparable mortalities for cancer of the stomach in Llanfyllin R.D. during the period 1953–59 were 581 (male) and 378 (female) deaths per million.

The representation of social class is given in Table XIII and Fig. 3 and 4. As in Llanfyllin R.D., the farming community is relatively more subject to cancer of the gastro-intestinal tract than to cancer of other sites. Farmers again show a

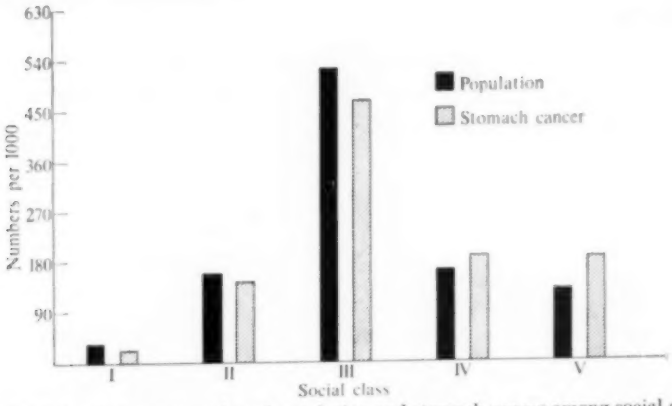


FIG. 1.—Proportionate representation of population and stomach cancer among social classes. *Note :* The population relates to the year 1951 and cancer to the years 1946–57. England and Wales.

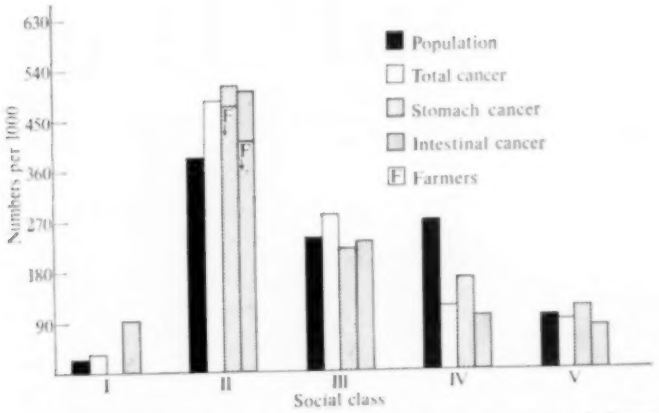


FIG. 2.—Proportionate representation of population and cancer among social classes. *Note :* The population relates to the year 1951 and cancer to the years 1946–59. Rural District of Llanfyllin.

high rate for stomach cancer but, when grouped with farm workers, their contribution to the total deaths from this cause is not much in excess of the joint proportion of these two occupational groups in the population.

Population and cancer distribution in the rural districts are similar both for the 1946–59 period in Llanfyllin R.D. and the 1953–59 period in Forden R.D. On the other hand Welshpool shows a distribution remarkably like that of England

and Wales (Fig. 1 and 4). Welshpool itself is about fifty per cent rural however, and when the total cancer deaths in the 1953–59 period are grouped strictly according to urban or rural residence, it is found that, while their general distribution is equal, there is a preponderance of deaths from stomach cancer among those with truly rural places of residence.

Cancer deaths in Welshpool, 1953–59

	Urban residence	Rural residence
All sites	38	39
Other cancer of digestive system	14	12
Stomach cancer	1	6

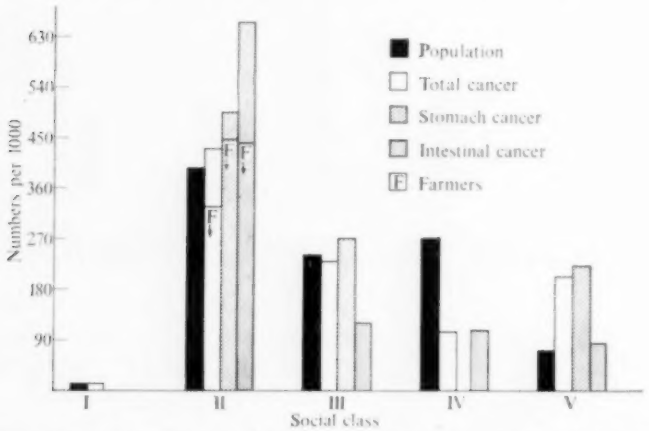


FIG. 3.—Proportionate representation of population and cancer among social classes. *Note*: The population relates to the year 1951 and cancer to the years 1953–59. Rural District of Forden.

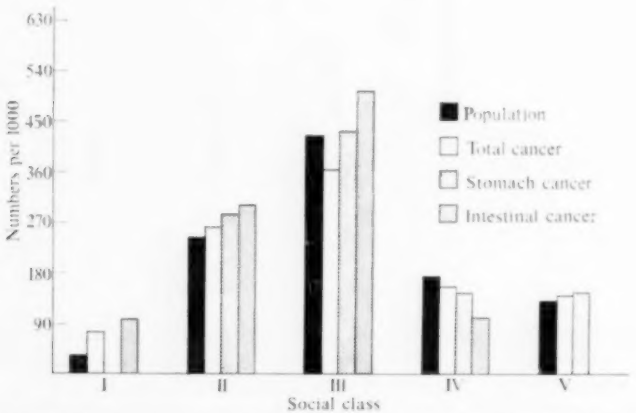


Fig. 4.—Proportionate representation of population and cancer among social classes. *Note*: The population relates to the year 1951 and cancer to the years 1953–59. Borough of Welshpool.

TABLE XII.—*Gastro-intestinal Cancer Mortality : Deaths per Million in the Districts of North Montgomeryshire in* 1953–59, (1954–59 *for the Boroughs of Montgomery and Llanfyllin*)

Site	Welshpool		Forden		Llanfyllin Borough		All areas except Llanfyllin R.D.	
	M.	F.	M.	F.	M.	F.	M.	F.
Oesophagus	—	46	288	—	—	237	137	45
Stomach	302	46	577	473	736	1425	434	354
Intestine	151	329	233	297	491	—	228	284
Rectum	151	46	346	178	—	237	206	133
Digestive system	1008	560	1615	1008	1963	2136	1347	927
All sites	2164	1590	2537	1722	2453	4751	2353	1990

TABLE XIII.—*Population and Cancer Distribution Among the Social Classes of the Four Areas (the Boroughs of Welshpool, Llanfyllin and Montgomery and Forden R.D.) : Population per* 1000 *occupied and Retired Males,* 1951, *and Corresponding Distribution for Cancer in* 1953–59 (1954–59 *for the Boroughs of Montgomery and Llanfyllin*)

	Social class				
	I	II	III	IV	V
Population	28	310	341	215	106
Cancer all sites	47 (9)	358 (69)	306 (59)	140 (27)	149 (29)
Farmers and farm workers	—	229 (44)	—	86 (17)	—
Cancer of stomach	29 (1)	429 (15)	257 (9)	86 (3)	199 (7)
Farmers and farm workers	—	371 (13)	—	86 (3)	—
Cancer of intestine	43 (1)	478 (11)	348 (8)	88 (2)	43 (1)
Farmers and farm workers	—	320 (8)	—	44 (1)	—

Note : The numbers in brackets refer to actual deaths.

Owing to the low numbers, undue significance cannot be attached to this finding, but it lends support to the association of stomach cancer with the more rural areas and perhaps with some widely-spread environmental factor. Possibilities of this nature will be discussed later. Histograms have not been included for the Boroughs of Llanfyllin and Montgomery owing to their small numbers. The addition of the Llanfyllin Borough mortality to that of the Rural District of Llanfyllin does not affect the latter materially, and a combination of the other three district mortalities is merely a compromise of the mortalities of Welshpool and Forden R.D.

Genetic considerations

In patients with stomach cancer blood group A was found to be commoner than in the general population (Aird *et al.*, 1953), and this excess of group A was thought to be associated only with carcinomata of the pyloric end of the stomach (Jennings, Balme and Richardson, 1956), and with blood group A1 rather than A2 (Walther, Raeburn and Case, 1956). The question arises as to whether or not an unusual distribution of these groups exists in Mid-Wales. In her paper given to the British Association in 1956 Dr. Ada C. Kopeć gave the A, B and O gene frequencies in thirteen regions of Great Britain including that of North Wales,

though not actually including Montgomeryshire. The gene A frequencies varied from 19·87 per cent in Western Scotland to 29·44 per cent in Sussex with North Wales occupying an intermediate position at 24·79 per cent. In another survey the gene O frequencies were found to rise on proceeding northwards and for similar latitudes they were higher in Wales than in Western England (Watkin, 1956). In the latter survey sub-group A2 was found to be unusually prevalent in Mid-Wales, being 35 per cent and nearly 50 per cent of group A in two population samples in North and Mid-Wales, compared with 22 per cent in Southern England. The geographical distribution of blood groups in the ABO system does not appear to influence in any way the incidence of stomach cancer in North Wales.

Geochemical considerations

Some of the more radioactive rocks in the British sedimentary sequence are to be found in North Wales. The highest uranium contents are to be found in certain black shales in rocks which include those of Upper Cambrian age (Ponsford, 1955). In the main the radioactivity is due to the content of uranium and it is customary to express the total radioactivity as uranium oxide equivalent. It has been found that the average shale is about four to six times as active as the average limestone. These black shales have been proved to be radioactive at all known localities but the activity and uranium enrichment are at the most only one quarter of those reported for the equivalent Swedish rocks. These rocks are to be found most prominently to the north and west of the Bala fault. To the south and east, which includes Montgomeryshire, the geological formation is largely of Silurian age and musuem specimens of black shales and phosphates of Silurian and Ordovician age have been found to possess a low radioactivity, though up to twice that of an average shale. The well-known phosphate deposit of comparable age at Pen-y-Garnedd in the Rural District of Llanfyllin is not radioactive (Wedd, *et al.* 1929). Great thicknesses of these black shales have not been adequately sampled however, and there is a possibility that other radioactive beds may be present.

The most obvious manner in which the radio-activity of these rocks could be further concentrated and communicated to populations would be through the drinking water. The distribution of uranium in a drainage system has been studied in the Fal River in Cornwall where it was found that the uranium oxide content in grams $\times 10^{-6}$ per litre rose from levels between 0·7 and 7·5 to levels between 1·0 and 25·0 after a prolonged rainy period which had been preceeded by several months of comparatively low rainfall (Ostle, 1954). The variation was attributed to the action of acidic water upon uranium followed by the leaching effect of subsequent sustained rainfall. As similar conditions were thought to apply in Mid-Wales, it was decided to investigate the water supplies of some of the places of residence where deaths from cancer of the stomach had occurred. The chief public water supplies of North Montgomeryshire were dealt with in like fashion. Using the quantitative field method for the determination of uranium in natural waters described by Ostle (1954), results were obtained which are given in Table XIV.

It is interesting to note that the average of the results shown in Table XIV is equivalent to 0·2 $\mu\mu$c, per litre (range 0·03 to 0·6 $\mu\mu$c per liter) as compared with 0·45 $\mu\mu$c per litre, 0·24 $\mu\mu$c per litre and 0·01 $\mu\mu$c per litre strontium-90 in lake, river and well water supplies respectively in 1957–58 (Stewart *et al.*, 1959). As

TABLE XIV.—*Uranium Oxide Content of Certain Water Supplies in North Montgomeryshire*

Code number	Locality	Type of source	Uranium oxide content
		Public supplies	
1	Llanerfyl	Springs	$0{\cdot}6 \times 10^{-6}$ g. per l.
2	Llanfair Caereinion	Banwy River	1·4 " "
3	Meifod	Springs	0·5 " "
4	Dolanog	"	0·6 " "
5	Llangynog	"	2·0 " "
6	Llanfyllin	"	0·6 " "
7	Llanfechain	"	1·1 " "
8	Llansantffraid	"	0·8 " "
9	Welshpool		
	(i) Trinity Well	"	0·9 " "
	(ii) Black Pools	Springs and surface water	0·6 " "
10	Foel	Springs	0·2 " "
11	Penybontfawr	"	0·1 " "
	Private residences where a death from cancer of the stomach had occurred		
12	Pen-y-Garnedd	Shallow well	$0{\cdot}6 \times 10^{-6}$ g. per l.
13	Hirnant	Springs	0·8 " "
14	Llanwddyn	Lake Vyrnwy	0·6 " "
15	Llanfihangel	Shallow well	0·9 " "
16	Bwlchycibau	" "	0·6 " "
17	Llanymynech	" "	0·9 " "
18	Guilsfield	" "	0·8 " "
19	Pentrebeirdd	Springs	0·9 " "
20	Llanfair Caereinion	Shallow well	0·9 " "
21	Penybontfawr	" "	0·2 " "
22	Pontrobert	" "	0·2 " "
23	Llandrinio	" "	0·7 " "
24	Llanfair Caereinion	" "	0·1 " "
25	Sarnau	" "	0·2 " "

Note : Sample numbers 10, 11, 18 and 21–25 were subject to repeat analysis by two different techniques and by two different analysts. Agreement was good considering the low content of uranium and in each case the greater of the two results is quoted.

far as the uranium content of the samples is concerned, serious significance can hardly be attached to such extremely small amounts of radiation especially as the uranium contents are no higher than would be expected in most natural waters. The highest bone activity due to strontium-90 in children under five years in 1956 was only one tenth of that due to natural radium when allowance had been made for the relative biological efficiency of the alpha rays, and it was about one thirtieth of the total natural dose to bone from internal and external sources (Bryant *et al.*, 1958*b*).

These results do not take into account the daughter elements however, or the great quantitative variation that may develop among them. Two examples of this variation, yet of different types, have been described recently in Devon and Wales. High activities of 0·1 $\mu\mu$c per ml. to 13·3 $\mu\mu$c per ml. have been recorded for waters in Devon (Abbatt *et al.*, 1960). These are presumably total radiations including beta as well as alpha activities and the thorium series as well as the uranium series ; nevertheless gamma spectrometry identified radon as being at least primarily responsible for the gamma activity of the samples. The ratio of thorium-232 to uranium-238 in a sample of Devon soil was 1·3 and the total

soil activity was about three times that of the samples from Wales (Mayneord, Turner and Radley, 1960). In Wales an aliquot of raw matt which showed increasing uranium series activity was found to have a lead-210 content twenty-seven times higher than the radium-226 level (Mayneord *et al.*, 1960). The difference between Wales and Devon as far as alpha activity is concerned is that, although in both Devon and Wales the soil showed small rises of 5 to 6 per cent in activity on sealing for about six months, in Wales and in Scotland the grass ash activities after six months' storage showed much higher values than in Devon due to the growth of polonium-210 (Mayneord *et al.*, 1960).

Two mechanisms are thought to be at work. The first is the leaching effect of underground waters of differing pH values upon uranium and thorium ores of varying qualities and of varying degrees of accessibility. Such waters, dependent also upon drainage and depth below the surface, may leach out certain members of the series more than uranium itself. In particular the radons-222 and -220 imprisoned in varying concentrations may be released in varying amounts. If small amounts of radium D (lead-210) were to be selectively released, the ratio of the disintegration constant of its main precursor radium-226 and its own constant (1 : 135) would ensure a similarly enhanced degree of radioactivity. The second mechanism is the retention of lead-210 from the atmosphere in proportion to rainfall after the fashion of the foliar retention of strontium-90. The basic uranium and thorium activities of soil samples in Wales were less than in Devon and certainly the uranium content of the water samples was lower so that it is possible that the second mechanism is of greater importance in Wales than in Devon. If a causative association is to be entertained between radioactivities and gastric cancer, then both of these mechanisms are likely to be relevant to the problem although, for reasons to be discussed, the second may be the more significant in Wales.

In Sweden, where radioactive shales are several times more active than in Wales, there is evidence of a gastric cancer mortality which is not only higher than the average but also constitutes a much great erproportion of cancer of the digestive system than in England and Wales. It must be admitted however that the high specific death rates for cancer of the stomach at ages over 75 years contributed substantially to the high total in Sweden (Table XV).

TABLE XV.—*Standardised Cancer Death Rates per Million of Population for Cancer of the Digestive System and Stomach only in Sweden and England and Wales*

	Male		Female	
	Cancer of digestive system and peritoneum	Cancer of stomach	Cancer of digestive system and peritoneum	Cancer of stomach
Sweden, 1951–55	—	433	—	322
1951–53	840	—	707	—
England and Wales, 1946–57	—	382	—	275
1951–53	953	—	800	—

Quantitative tests were carried out on the public water supplies in order to estimate the fluoride content and in each case the amount was negligible or of the order of 0·1 parts per million.

DISCUSSION

The mortality from cancer of the stomach in England and Wales has been declining over the period 1936–54 but not in the age-groups over 75 years. On the other hand the comparative mortality index for Wales rose by 5·4 per cent (male) and 5·6 per cent (female) between the quinquennia 1946–50 and 1951–55. Again, Wales differed from England and Wales in having a higher standardised mortality ratio for stomach cancer in rural than in urban areas (General Register Office, 1957). This feature of the mortality can be seen in miniature in the Welshpool figures of the present study. The latest standardised mortality ratios for Wales (for 1958) are 130 (male) and 159 (female) compared with 93 and 107 respectively for cancer of all sites (Registrar General, 1958).

The pattern of gastric cancer appears to be related to geographical regions such as Wales and the Northern region of England. The high rates prevailing in these areas must have contributed appreciably to the fact that England and Wales had the fifth highest mortality from cancer of the digestive organs and peritoneum during the years 1951–53 in a list of twentyone countries. (The death rates were made available by the Institute of Cancer Research.) Germany, Switzerland, Scotland and France occupied the first four consecutive places on this list followed by England and Wales, Denmark, Finland, Norway, Sweden and Ireland. It must be observed that all these countries except Finland had older populations than the remaining eleven countries in which the populations over the age of 50 years amounted to less than 18 per cent. Finland, Japan and Chile had fairly high specific rates for the ages 50–65 however, and apart from this broad division of the countries into two groups, there was no detailed correlation between the numbers of old people and the mortalities. Local investigations within countries, whether statistical or otherwise, are more likely to reveal possible causal relationships than the rather unwieldy national statistics. Cancer of the stomach for females in Wales for the years 1947–53 has been accorded such treatment by the geographer, and his map of Wales shows that nearly all of the rural counties including Montgomeryshire have standardised mortality ratios ranging from one and three-quarters to twice the national average : in fact the map focuses attention upon rural districts *per se* because so many of the small urban districts within the large rural districts, including those in Montgomeryshire, have lesser mortalities (Howe, 1959). The annual report of the Medical Officer of Health for Carmarthen (Evans, 1959) contains statistics which show that the gastric cancer death rate for that county was 486 per million or 23·6 per cent of the total cancer death rate. In 1958, stomach cancer in England and Wales amounted to 15·6 per cent (male) and 13·7 per cent (female) of the total cancer deaths (Registrar-General, 1958).

Investigations have now been carried out on an even more local basis by means of the analysis of soil samples from the gardens of houses where a case of stomach cancer was known to have occurred, comparing the results thus obtained with those of control samples. In Caernarvonshire, Merionethshire and Cheshire, garden soils taken from houses where a person has died of stomach cancer after fifteen years or more of residence, have been found to have higher median concentrations of zinc and chromium than garden soils from houses in the same counties where a person has died from a cause other than cancer or has died of cancer after residence of less than two years. In Welsh districts with high stomach mortality, but not elsewhere, the incidence of gastric cancer after 10–19 years of

residence was also found to be excessive where the garden soil had a high organic carbon content and even more so after 20 years or more of residence where the soil had a medium organic carbon content (Stocks, 1958). Zinc is one of the trace elements shown to be essential for the higher forms of animal life, but chromium is not even among the apparently unessential elements known to be normally present in the body tissues (Underwood, 1956). It was therefore all the more significant that a local high gastric cancer rate in a township in Devon, situated in a district which had an average gastric cancer rate, was found to be associated with a high chromium content of the garden soils of the houses in which a case of stomach cancer had occurred. Furthermore the excess of chromium, zinc, cobalt, nickel and organic matter at the stomach cancer addresses exceeded the respective control levels to a significant degree in two sub-districts in which there were tanneries (Stocks and Davies, 1960*a*). Both tanneries used chrome and the deaths occurred among those who might have been expected to eat much of their own garden produce (Nicholson, 1960). A positive association has also been found between chromium content of the soil and intestinal cancer (Stocks and Davies, 1960*b*), but in the Rural District of Llanfyllin no relationship between intestinal cancer and the occupation of tanner or the existence of tanneries was found. Samples of soil of course might have revealed high chromium contents. The importance which might be attached to micronutrient deficiencies or to carcinogenic contaminants of vegetable foods in North Wales (Stocks, 1959; Davies and Griffith, 1954; Legon, 1952) has been doubted on the grounds that, with the exception of potatoes, 78–85 per cent of vegetables are brought into North Wales (Howe, 1959).

Whether this mode of direct conveyance of carcinogens to the body by vegetable foods is important or not, many workers have concentrated attention upon the other and main component of the diet, namely water. London boroughs supplied by well-water had lower cancer mortalities than most of the boroughs supplied by river water (Stocks, 1947). Similar findings in the Netherlands stimulated work on soil analysis there (Tromp and Diehl, 1955), yielding results not unlike those in North Wales (Davies and Griffith, 1954; Stocks, 1958). In both cases the higher gastric mortalities were associated with acid poorly drained peaty soils of the type likely to produce waters having a solvent action in piped supplies. Treated and untreated water supplies in rural Wales are generally soft or moderately so and, with several exceptions, the softest waters occur in the highest gastric cancer-bearing areas (Howe, 1959). In Montgomeryshire the southern half of the county has fairly hard water supplies although the raised gastric cancer mortality applies generally to the rural districts throughout the county. The water supplies in the Llanfyllin R.D. are mostly soft apart from two treated supplies whose treatment includes passage through a contact chamber of limestone chippings. Despite a gradual improvement since 1946 in the provision of public mains in this district, the section of the population served in this way in 1957 was still only 38·7 per cent. Many of the public supplies are virtually untreated waters which nevertheless reach the necessary bacteriological standards for drinking water. An attempt was made to ascertain differences in gastric cancer mortality related to public or private water supplies but none were found. It should be noted however that Liverpool's water supply is derived from Lake Vyrnwy whose catchment area extends over much of the rural district of Llanfyllin, but in this case an excess of stomach cancer in Liverpool appears only amongst females (S.M.R. 128 in 1950–54), and of course the water is subjected to the full treatment

process not far from the city. A negligible amount of water from Lake Vyrnwy is used in Llanfyllin R.D. itself. As an indication of the potential contamination of private supplies in this district 20 out of 29 samples were found to be either unsatisfactory or suspicious on bacteriological examination in 1957 compared with 10 out of 205 samples from treated public supplies and 59 out of 318 samples from untreated public supplies. These private supplies were usually tested after protective works had been carried out in preparation for a grant-aided farm water scheme or the erection of a new house, so that the results may have indicated better conditions than actually prevailed. Recent experimental work justifies alertness to the possibilities of water contamination. Because it was thought possible that even weakly acting carcinogenic agents ingested over a long period might be dangerous, mice were fed 25 μg. 3,4-benzopyrene for 400 days with or without surface-acting agents. Definite carcinoma of the stomach was produced only in the group of mice which had been fed with a normal diet but had received benzopyrene in their drinking water to which detergent had also been added (Borneff, 1960).

Mutagens, which have much in common with the chemical carcinogens, also merit some consideration. Whilst the fall-out of strontium-90 cannot be expected to have a significant bearing, if any at all, upon the present study, the numerous papers on strontium-90 provide valuable information much of which can be applied to the allied subject of natural fall-out. In the breakdown of uranium the emanation of radium (Radon-222) escapes from rock and soil in varying degrees throughout the world. The rate of escape of this element is highest in the dry equatorial regions from which it tends to rise with upward air currents towards the stratosphere. The rapid change through four radioelements is over 90 per cent complete in about two weeks with the formation of lead-210 which has a half-life of nearly 19·5 years. Being particulate, this element reaches the ground as fall-out in temperate regions after approximately four weeks in the atmosphere, a period sufficient for appreciable atmospheric mixing to occur, yet short compared with its radioactive half-life (Burton and Stewart, 1960). The model of global circulation of air (Dobson, 1956) and the world-wide measurement of long-lived fission products (Stewart *et al.*, 1957) help to explain the general pattern of deposition which, although more consistently related to the concentration of these products in air or rain, is in the final distribution roughly proportional to rainfall (Peirson, Crooks and Fisher, 1960). The areas of rainfall in the United Kingdom can be divided as follows :—

Over 60 inches :	West Highlands
	Much of Wales
	Much of Cumberland
	Three small areas in Devon
30–60 inches :	Western half of Great Britain
	Northern Ireland
Under 30 inches :	Eastern half of Great Britain

The high level of basic top-soil activity in Wales, coupled with the restricting influence of an even moderate rainfall upon the rate of escape of radon-222 from the surface of the soil (Jaki and Hess, 1958) accentuate the effects of rainfall itself. The lead-210, whose content in one matt specimen was found to be twenty-seven times higher than the radium-226 level, eventually becomes polonium-210 with a half-life of 138·7 days and a radiation of the alpha type. Polonium-210 forms

the chief natural activity in pasture plants, in fact about 84 per cent (Mayneord *et al.*, 1960).

It is relevant to compare strontium-90 and lead-210 deposition. In 1956–57 the deposition of strontium-90 was 2600 $\mu\mu$c per square metre at Milford Haven. The deposition of lead-210 was calculated to be 2260 $\mu\mu$c. m^2 per per year per 100 cm. rainfall, which might mean anything from 3000 to 4000 $\mu\mu$c./m in the upland areas of Wales (Burton and Stewart, 1960). Strontium-90 is particularly well entrapped in the slow-growing matted pastures characteristic of the relatively unproductive hill areas. The soil is uncultivated and peaty and there is a low yield of vegetation and the strontium-90 tends to remain substantially in the topsoil for years so that the uptake of these elements by animals is bound to be high. In 1956 the measurements per square metre of the surface and per gramme calcium in soil and grass were all many times higher in these upland areas than elsewhere. It was not surprising therefore to find readings of 183 and 35 $\mu\mu$c. per g. calcium in sheep bone at Cwmystwyth and Welshpool respectively, in contrast to readings of 7 to 15 $\mu\mu$c. per g. calcium for lowland sheep (Bryant *et al.*, 1958*a*, 1958*b*). Total soil alpha activities were found to be 15 times higher in Wales than in South-East England and the total grass ash alpha activities were also many times higher (Mayneord *et al.*, 1960). As the proportion of strontium-90 per gramme calcium is about 100–300 times higher in grass grown on calcareous soil than in the soil itself, the dominant mode of contamination is by means of foliar retention (Bryant *et al.*, 1958*a*, 1958*b*). This is also borne out by the ratios of strontium-90 to stable strontium and calcium in flour, which were consistently lower than those for bran which is more accessible to current fall-out (Agricultural Research Council, 1958). The same also applies to polonium-210 because the total alpha activities for the outer parts of the plant such as the leaf and bark have been found to be much higher than those for the fruit and root (Mayneord *et al.*, 1960). Despite the fact that the thorium series contributes more than the uranium series to soil activity—and in Wales soil samples were found to have Th/Ur ratios of about 4·5—the plant has a preference for the radium isotopes which have been found to contribute up to 90 per cent of the total alpha activity in the plant (Mayneord *et al.*, 1960). Just as a biological discrimination against strontium-90 may amount to a factor of 25 to 30 from vegetation to the bone of a child (Bryant *et al.*, 1958*a*), there is also likely to be discrimination against polonium. The difference between readings in samples of dry grass (2·6 to 26 $\mu\mu$ c. per gramme dry grass) and in samples of lamb kidney (of which the highest reading was 2·38 $\mu\mu$c. per gramme wet cortex tissue) in two Welsh towns, gives a factor of anything between 1 and 11 (Hill, 1960). Such low discrimination is of course balanced by the shorter half-life and lower body retention of polonium.

The level of activity on entry to the food chains is likely to be much higher in Wales due to the greater rainfall and cumulative effects. Because foliar retention is so much greater it was not surprising to find that only a short period of time elapsed between the deposition of strontium-90 and its appearance in milk. Measurements of strontium-89 in relation to strontium-90 in milk and rainwater confirmed that this period was only about one or two months. In 1956 the amount of strontium-90 in milk (4·4 $\mu\mu$c. per litre and also per gramme calcium) was nearly twice that found in rainwater (Bryant *et al.*, 1958*b*; Bryant, Morgan and Spicer, 1958). The ratios of strontium-90 to calcium in blood and milk of animals were found to be respectively 0·25 and 0·12 of the ratio in the diet. Slaughtered

animals which have been raised on dairy pastures will have twice the ratio of strontium-90 to calcium as that found in milk. At stations in Cardiganshire and Merionethshire in 1959 however, the proportions of strontium-90 to calcium in the milk were 33 and 49·9 $\mu\mu$c. per gramme calcium respectively. Where beef cattle, grazed on acid unimproved upland soils, are concerned, and even more so in relation to sheep, high strontium-90 readings are to be expected in the beef and mutton. In 1959 meat only accounted for about 3·5 per cent of the strontium-90 in the human diet in the United Kingdom (Agricultural Research Council, 1959), but a tenfold increase in this proportion, as might occur in Welsh meat, could increase the total intake by a quarter. In Wales as elsewhere milk is mainly derived from dairy farms situated in the more fertile lowland areas in Wales and Western England. Meat on the other hand is more likely to be derived from hill cattle and sheep. In Llanfyllin R.D. local slaughtering is common although in border areas meat also tends to be bought " off the hook " from centres such as Shrewsbury. Polonium-210 may therefore gain access to the body disproportionately through meat in Welsh districts.

The alpha activities of certain foodstuffs have been ascertained (Mayneord, 1959). In some cases the figures obtained can be compared directly with the beta activities of strontium-90 in 1959, but no account has been taken either of the tenfold relative biological efficiency of the alpha radiation or the concentration of strontium-90 in bone (Table XVI). It has been estimated that over 96 per cent of

TABLE XVI.—*Radioactivities of Certain Foodstuffs*

Foodstuff	Maximum alpha activity ($\mu\mu$c. per 100 g.)	Activity due to strontium-90 (year 1959) ($\mu\mu$c. per 100 g.)
Cereals	60	—
Cereal-based infant foods	—	4·1
Teas	40	39·7
Flours	14	1·56
Wholemeal flour	—	3·8
Milks (evaporated)	1 to 2	2·3
Eggs	0·9	0·52
Vegetables	0·7	1·0

the total strontium-90 intake is dietary (Agricultural Research Council, 1959). The daily alpha activities of diets, though of a small order, may vary, according to the foods chosen, by a factor as high as a thousand (Mayneord, 1959). Having gained access to the body it would have to be supposed that polonium-210, and for that matter any carcinogenic agent, would undergo some form of concentration in the stomach, perhaps in the cancer-prone pyloric region. Studies of the distribution of polonium in animals and in the human body did not reveal any abnormal concentration in the stomach, but the amounts of polonium being administered were almost of pharmacological proportions (200–300 $\mu\mu$c. per g. body weight) compared with the amounts being considered here. There were high concentrations in the liver and kidney however, the latter being associated with clearances that were considerably lower than for some similar metals (Fink, 1950).

Two characteristics of polonium compounds are that they are not ionised and that they can separate in the colloidal state (Sidgwick, 1950). Such compounds might be expected to show resemblances to the other members of their chemical

sub-group which includes sulphur and selenium. Most of the selenium in wheat is associated with the protein and is therefore well distributed throughout the grain especially in the bran. In organic form in cereal grain it is highly toxic and it is thought that it may react with the sulphur-containing amino acids, partially replacing the sulphur. In animal tissues it concentrates in liver, muscle and the gastro-intestinal tract (Underwood, 1956). Just as cobalt forms four per cent of the molecule of vitamin B_{12}, so polonium might enter the body in similar fashion or, like selenium, it might supplant other elements in the enzyme systems, especially the cytochrome system which may govern the production of hydrochloric acid in the stomach. The radioactivity of polonium-210 might be expected to enhance or exceed the effect of any toxicity inherent in the element itself.

Cellular injury initiates growth and repeated injury may profoundly alter the processes both of agamic reproduction and of regeneration. Hyperplasia blends imperceptibly into neoplasia like graded manifestations of tissue malformations (Smithers, 1959). Carcinogenesis also appears to have an initiating phase and a promoting phase with most of the chemical carcinogens combining both phases. The cellular change induced by an initiating substance is irreversible, instantaneous and invisible (Berenblum and Shubik, 1949). A somatic mutation, such as might be caused by an alpha radiation is likely to produce the first of these phases and bring on the second phase more rapidly than other forms of cellular injury. It is certain that only microgramme quantities of the more active carcinogenic compounds are required to produce tumours in mice and human cancer also may be produced either by small amounts of highly active compounds, perhaps acting for a relatively short time, or larger amounts of weaker agents acting over many years (Cook, 1957).

The finding of an increased mortality for cancer of the intestine in the Llanfyllin R.D. is contrary to expectations because in 1950–54 the standardised mortality ratios for cancer of the intestine and rectum in the rural districts of Wales were only 96 (male) and 105 (female) (General Register Office, 1957), and in 1950–52 in the three counties of Montgomeryshire, Cardiganshire and Breconshire they were 90 (male) and 94 (female) (Griffith, personal communication). In view of the statistical evidence against an increased intestinal cancer death rate in Wales, the figures given for Llanfyllin R.D. cannot attract other than sceptical comment owing to the small numbers involved ; nevertheless the excess was of similar proportions to that for the stomach over the same period. The extension to the intestine of the same carcinogenic influence that makes stomach cancer mortality excessive in Wales is possible ; indeed it is perhaps surprising that in Wales the whole of the digestive system is not equally affected.

The validity of the diagnoses on the death certificates in North Wales is such that there has been no over-statement of stomach cancer mortality and the incidence of the disease is no higher amongst people of Welsh parentage than amongst those of English parentage living in the same districts (Stocks, 1958). In Llanfyllin R.D. itself no evidence was found that certification had been inaccurate either recently or twenty to thirty years ago.

SUMMARY

The study of gastric cancer mortality in North Montgomeryshire commenced with the Llanfyllin R.D., being the largest of the five constituent authorities, but

eventually it was extended so as to cover all five authorities. The total cancer rate for the Llanfyllin Rural District during the period 1946–59 only slightly exceeded that for England and Wales during an almost similar period. The excess would have been greater but for the high rates for lung cancer in males and cancer of the genital system in females in England and Wales. When the cancer sites were examined individually however, it became obvious that this district, in common with the rest of rural Wales, possessed a high mortality rate for gastro-intestinal cancer, high enough in fact to off-set the national rates for male lung cancer and female genital cancer. The standardised mortality ratios in Llanfyllin R.D. were found to be 135 (male) and 161·5 (female) during the period 1946–59. The apparent disparity between the sexes was due largely to the excess of older men in the community, thus strengthening the view that a general carcinogenic agent might be at work.

There was a suggestion of earlier death from this cause but no specific age group or occupation was involved in the increase and the social variations tended to strike a balance between stomach and intestinal cancer so that the total for both was evenly distributed over all the social classes. Farmers in the district, in common with farmers in England and Wales, showed a small excess of mortality for stomach cancer in relation to the others in their social class ; hence the excess of mortality for Social Class II in relation to its population. The finding of an almost equally high rate for cancer of the intestine was unexpected in the light of the normal standardised mortality ratios which have been recorded for this site in Wales. The standardised mortality ratios for this site in Llanfyllin R.D. were 142·9 (male) and 149·8 (female).

The other four districts, with the exception of the relatively urban areas, were also found to have high gastric cancer mortality ratios. In one of the boroughs however, there was a suggestion that most of the stomach cancer deaths occurred at strictly rural residences, although otherwise this town showed a representation of population, social class and stomach cancer mortality not unlike that for England and Wales.

The discovery of a common dietary factor is not easy. Recent experimental work justifies alertness to the possibilities of water contamination and in the Llanfyllin R.D. much of the drinking water is virtually untreated though passing the necessary bacteriological standards ; furthermore it is derived from poorly drained peaty soils. Knowing that the Silurian formation of Mid-Wales possesses a significant degree of radioactivity, especially in certain shale beds, the public water supplies and many private supplies from houses where there had been a stomach cancer death were tested quantitatively for uranium oxide. The results showed no correlation with the cancer residences and their average, which was $0{\cdot}7 \times 10^{-6}$ g. per litre, was not considered to be higher than would be expected in most natural waters.

The possible significance of the uranium daughter elements is discussed in the light of the knowledge gained from studies of the fall-out of fission products. In 1956–57 the deposition of strontium-90 was 2600 $\mu\mu$c per square metre at Milford Haven compared with 2260 $\mu\mu$c per square metre for lead-210 which is the chief daughter element involved in natural fall-out. The alpha activities of these elements and the strontium-90 beta activities of certain foodstuffs were also found to be rather similar. As the dominant mode of contamination is by foliar retention and as samples of grass matt in Wales have been found to contain

polonium-210 in amounts many times greater than those found in South East England, it is suggested that this element may gain access to the human body disproportionately through beef and mutton. These foodstuffs are likely to be derived from hill cattle and sheep which have grazed widely upon upland pastures of poor quality and in the area of the present study local slaughtering has always been the custom. Detailed measurements of the radioactivity of water supplies like those being made at present in West Devon, but also including meat supplies, should yield further information of value.

The gene frequencies of Montgomeryshire were not considered to have any bearing upon the incidence of stomach cancer.

I wish to express gratitude to those who helped me in this investigation: to Mr. S. H. U. Bowie, the Chief Geologist of the Geological Survey who undertook to provide the uranium oxide analyses; to the Institute of Cancer Research whose statistics on Sweden and other countries were made available; to my colleagues and members of my staff.

REFERENCES

Abbatt, J. D., Lakey, J. R. A. and Mathias, D. J.—(1960) *Lancet*, ii, 1272.
Agricultural Research Council.—(1958) *Radiobiol. Lab. Rep.* No. 1.—(1959) *Ibid.*, No. 2.
Aird, I., Benthall, H. H. and Roberts, J. A. F.—(1953) *Brit. med. J.*, i, 799.
Berenblum, I. and Shubik, P.—(1949) *Brit. J. Cancer*, **3**, 109.
Borneff, J.—(1960) *Bull. Hyg.*, **11**, 1077.
Bryant, F. J., Chamberlain, A. C., Morgan, A. and Spicer, G. S.—(1958*a*) *Atomic Energy Res. Estab.*, HP/R 2056.—(1958*b*) *Ibid.*, HP/R 2353.
Idem, Morgan, A. and Spicer, G. S.—(1958) *Ibid.*, HP/R 2730.
Burton, W. M. and Stewart, N. G.—(1960) *Nature, Lond.*, **186**, 584.
Cook, J. W.—(1957) *Lancet*, i, 333.
Davies, R. I. and Griffith, G. W.—(1954) *Brit. J. Cancer*, **8**, 56.
Dobson, G. M. B.—(1956) *Proc. Roy. Soc.*, **236**, 187.
Evans, R.—(1959) *Rep. Minist. Hlth*, Carmarthenshire, p. 18.
Fink, R. M.—(1950) 'Biological Studies with Polonium, Radium, and Plutonium.' New York, London (McGraw-Hill).
General Register Office.—(1957) 'Studies on Medical and Population Subjects,' No. **13**, pp. 5, 23.
Idem.—(1951) Census. County Report (Merionethshire, Montgomeryshire, Radnorshire), pp. 70, 77.
Hill, C. R.—(1960) *Nature, Lond.*, **187**, 221.
Howe, G. M.—(1959) *Brit. J. prev. soc. Med.*, **13**, 204.
Jaki, S. L. and Hess, V. F.—(1958) *J. geophys. Res.*, **63**, 373.
Jennings, D., Balme, R. H. and Richardson, J. E.—(1956) *Lancet*, ii, 11.
Legon, C. D.—(1952) *Brit. med. J.*, ii, 700.
Mayneord, W. V.—(1959) *Roy. Soc. Hlth J.*, **79**, 338.
Idem, Turner, R. C. and Radley, J. M.—(1960) *Nature, Lond.*, **187**, 208.
Nicholson, G.—(1960) *Publ. Hlth, Lond.*, **74**, 403.
Ostle, D.—(1954) *Min. Mag.*, **91**, 201.
Peirson, D. H., Crooks, R. N. and Fisher, E. M. R.—(1960) *Atomic Energy Res. Estab.*, R.3358.
Ponsford, D. R. A.—(1955) *Bull. geol. Surv. G.B.*, No. 10, p. 24.

REGISTRAR GENERAL.—(1951) *Decennial Supplement, England and Wales*, Occupational Mortality, Part I.—(1953) *Statistical Review of England and Wales*, Text Volume (Appendix B), 247.—(1954) *Ibid.*, Commentary, p. 135.—(1958) *Ibid.*, Commentary, pp. 127, 129, 131.

SIDGWICK, N. V.—(1950) 'The Chemical Elements and Their Compounds.' London (Oxford University Press).

SMITHERS, D. W.—(1959) *Lancet*, i, 589.

STEWART, N. G., OSMOND, R. G. D., CROOKS, R. N. AND FISHER, E. M. R.—(1957) *Atomic Energy Res. Estab.*, HP/R 2354.

Idem, CROOKS, R. N., OSMOND, R. G. D., OWERS, M. J. AND HEALY, C.—(1959) *Ibid.*, HP/R 2795.

STOCKS, P.—(1947) 'Regional and Local Differences in Cancer Death Rates.' London (H.M. Stationery Office).—(1958) *Rep. Brit. Emp. Cancer Campgn.* **35**, Supplement.—(1959) *Brit. med. J.*, i, 74.

Idem AND DAVIES, R. I.—(1960*a*) *Publ. Hlth, Lond.*, **74**, 408.—(1960*b*) *Brit. J. Cancer*, **14**, 8.

TROMP, S. W. AND DIEHL, J. C.—(1955) *Ibid.*, **9**, 349.

UNDERWOOD, E. J.—(1956) 'Trace Elements in Human and Animal Nutrition.' London (Academic Books).

WALTHER, W. W., RAEBURN, C. AND CASE, J.—(1956) *Lancet*, ii, 970.

WATKIN, I. M.—(1956) *Heredity*, **10**, 161.

WEDD, C. B., SMITH, B., KING, W. B. R. AND WRAY, D. A.—(1929) *Mem. geol. Surv. U.K.*, p. 52.

THE INCIDENCE OF CARCINOMA IN PATIENTS DYING FROM LEUKAEMIA, MALIGNANT DISORDERS OF PLASMA CELLS, AND MALIGNANT LYMPHOMA

J. S. CORNES, T. G. JONES AND GLORIA B. FISHER

From the Vincent Square Laboratories, Westminster Medical School, the Department of Morbid Anatomy, the Postgraduate Medical School, and the Research Department, St. Mark's Hospital, London

Received for publication February 8, 1961

In a recent survey of 40 consecutive cases of primary malignant lymphoma of the small and large intestine seen in the Vincent Square Laboratories of Westminster Medical School and the Research Department of St. Mark's Hospital, London, there were 5 carcinomas, an incidence of 12·5 per cent. Some data concerning the 40 cases of primary malignant lymphoma of the intestinal tract are given in Table I, and brief details of the 5 carcinomas are given in Table II.

TABLE I.—*Incidence of Carcinoma in Patients with Primary Malignant Lymphomas of the Small and Large Intestine*

Cell type	Number of patients	Number of patients with carcinoma	Percentage of patients with carcinoma
Hodgkin's disease	5	0	12·5
Reticulum cell sarcoma	14	1	
Giant follicle lymphoma	1	0	
Lymphosarcoma	20	4	
Total	40	5	

Detailed accounts of four of the five carcinomas have been published elsewhere (Cornes, 1960, 1961). In a further study of 100 cases of benign lymphoma of the rectum there were seven carcinomas, an incidence of 7 per cent (Cornes, Wallace and Morson, 1961). The large number of cases in these two series reflects the specialised work of the St. Mark's and Gordon Hospitals in the surgical treatment of intestinal disorders. Since more than half the patients in these two series are still alive the incidence of carcinoma may eventually prove to be higher than that stated. We were therefore interested to compare these findings with the incidence of carcinoma in patients dying from leukaemia or from generalised malignant tumours of lymphoid tissue.

Slaughter (1944) and Warren and Ehrenreich (1944), in their studies of multiple malignancy, concluded that multiple primary cancers arose more frequently than would be expected by chance alone. However, Morrison, Feldman and Samwick (1944) found only two instances of carcinoma in a series of 600 patients suffering from leukaemia, and Mider, Schilling, Donovan and Rendall (1952) found only 16 primary cancers in 519 patients with leukaemia or malignant lymphoma. Videbaek (1947) in a study of 209 patients with leukaemia, 4041 of their relatives, and 3641 controls, found a higher incidence of cancer in the leukaemia families

than in the control population, and Beresford (1952) found 19 cases of carcinoma in 106 patients with chronic lymphatic leukaemia. Moertel and Hagedorn (1957) reviewing 194 cases of leukaemia and malignant lymphoma with co-existent primary malignant lesions reported in the literature, and 120 similar cases seen at the Mayo Clinic, concluded that the incidence of another primary malignant lesion in patients with leukaemia or malignant lymphoma was probably comparable to, and perhaps exceeded, that in any segment of the general population of similar age. Weitzel (1958) in a survey of 3398 patients dying from carcinoma, and 365 patients dying from lymphoma or leukaemia, who came to autopsy at the Mallory Institute of Pathology in Boston, found that the incidence of multiple primary carcinomas was 4·2 per cent and the incidence of carcinoma co-existent with lymphoma and leukaemia was 8·0 per cent. A striking feature of this series was the finding of 11 carcinomas with 57 cases of malignant disorders of plasma cells, an incidence of 19·3 per cent.

Because of the general lack of agreement between the series reported in the literature we decided to find out the incidence of carcinoma in patients dying from leukaemia, malignant disorders of plasma cells, and malginant lymphoma, who came to autopsy at the Westminster and the Postgraduate Medical Schools of London University.

MATERIALS AND METHODS

This study was based on 9383 autopsies performed at the Postgraduate Medical School, London, between April 1935 and October 1960, and on 5561 autopsies performed at the Westminster Medical School between 1938 and October 1960. The autopsies were performed to determine the causes of death, to assess the nature and extent of the principal diseases, and to detect any associated disorders. Amongst these 14,944 autopsies there were 585 cases of leukaemia, multiple myeloma, and malignant lymphoma. The clinical records and post-mortem reports of the 585 cases were studied to find out the number of patients with carcinoma. The diagnosis of leukaemia was based on a study of peripheral blood films and bone marrow biopsies which were taken in every case, and as far as possible we have tried to eliminate all leukaemoid reactions. For the sake of convenience the subacute and acute forms of leukaemia have been considered together, and the monocytic leukaemias include both the Naegeli and the Schilling types. The diagnosis of multiple myeloma and malignant lymphoma was based on the histological

TABLE II.—*Details of 5 Carcinomas found in Patients with Primary Malignant Lymphoma of the Intestinal Tract*

Patient		Carcinoma			Type of malignant lymphoma	Treatment of malignant lymphoma	Relation of carcinoma to lymphoma
Age	Sex	Site	Type	Metastases			
57	M.	Rectum	Adenocarcinoma	0	Lymphosarcoma	Resection and radiotherapy	Simultaneous.
52	,,	Colon	,,	0	,,	Ditto	Followed—1 year.
52	,,	Colon and rectum	Adenocarcinoma (multiple)	+	Reticulum cell sarcoma	,,	Followed—20 months.
66	,,	Prostate	Adenocarcinoma	0	Lymphosarcoma	None	Simultaneous.
48	,,	Bronchus	Anaplastic carcinoma	+	Lymphosarcoma	Resection and radiotherapy	Followed—15 years.

study of biopsies taken in life, and on the findings at autopsy. The classification of the malignant lymphomas used in this study corresponds with that given by Harrison (1960). Sections of all the carcinomas were available for study, and the clinical diagnoses were confirmed histologically in every case. Apart from leukaemia, multiple myeloma and malignant lymphoma, no other type of sarcoma was found in this series.

RESULTS

The incidence of carcinoma in patients dying from leukaemia, malignant disorders of plasma cells, and malignant lymphoma, is shown in Table III. Amongst 265 cases of leukaemia three were 9 carcinomas, an incidence of 3·4 per cent. Amongst 43 cases of malignant disorders of plasma cells there were 2 carcinomas,

TABLE III.—*Incidence of Carcinoma in Patients Dying from Leukaemia, Malignant Disorders of Plasma Cells, and Malignant Lymphoma*

Cell type	Number of patients	Number of patients with carcinoma	Percentage of patients with carcinoma
Acute myeloid leukaemia	54	2	2·7
Acute lymphatic leukaemia	48	0	
Acute monocytic leukaemia	32	1	
Stem cell leukaemia	13	1	
Chronic myeloid leukaemia	56	1	3·4
Chronic lymphatic leukaemia	58	3	
Chronic monocytic leukaemia	1	0	
Plasma cell leukaemia	2	1	4·6
Multiple myeloma	41	1	
Hodgkin's disease	132	1	2·1
Reticulum cell sarcoma	91	4	
Giant follicle lymphoma	8	0	
Lymphosarcoma	49	1	
Total	585	16	2·7

an incidence of 4·6 per cent ; and amongst 280 cases of malignant lymphoma there were 6 carcinomas, an incidence of 2·1 per cent. The total number of carcinomas found in the 585 cases was 16, giving an overall incidence of 2·7 per cent.

Brief details of the 16 cases with carcinoma are given in Table IV, the age given being that at the time of diagnosis of the leukaemia, malignant lymphoma or multiple myeloma. In 8 cases the carcinomas developed before the onset of leukaemia or malignant lymphoma. In 3 cases the carcinomas apparently developed simultaneously with the onset of malignant disorders of plasma cells or malignant lymphoma. In the remaining 5 cases the carcinomas developed after the onset of leukaemia or malignant lymphoma. At autopsy carcinomatous metastases were found in only 5 cases.

DISCUSSION

The autopsy services of the Westminster Medical School and the Postgraduate Medical School serve busy general hospitals caring for every type of patient. Both centres are distinguished for their treatment of malignant disease, and any

TABLE IV.—*Details of 16 Carcinomas found in Patients Dying from Leukaemia, Malignant Disorders of Plasma Cells, and Malignant Lymphoma*

Patient		Carcinoma						
Age	Sex	Site	Type	Metastases	Treatment of carcinoma	Type of lymphoma or leukaemia	Treatment of lymphoma or leukaemia	Relation of carcinoma to lymphoma or leukaemia
70	M.	Skin	Basal cell ca.	0	Radiotherapy	Acute myel. leuk.	Chemotherapy and steroids	Preceded—9 years.
44	F.	Ovary	Medullary ca.	0	Excised	,, ,, ,,	Ditto	Preceded—5 years.
53	,,	Breast	Adenocarcinoma	+	,,	Acute mono. leuk.	None	Preceded—15 months.
78	,,	Rectum	,,	0	,,	Stem cell leuk.	Steroids	Preceded—20 years
38	M.	Bronchus	Malig. adenoma	+	Radiotherapy	Chr. myel. leuk.	Radiotherapy Chemotherapy	Followed—21 months.
66	F.	Stomach	Adenocarcinoma	0	Excised	Chr. lymph. leuk.	Radiotherapy	Preceded—4 years.
49	,,	Cervix	Sq. cell ca.	0	Radiotherapy	,, ,, ,,	Radiotherapy Chemotherapy and steroids	Followed—8 years.
64	M.	Oesophagus	,, ,, ,,	+	None	,, ,, ,,	None	Followed—2 months.
58	F.	Colon	Adenocarcinoma	0	,,	Plasma cell leuk.	Chemotherapy and steroids	Simultaneous.
72	M.	Bronchus	,,	+	,,	Multiple myeloma	None	,,
51	,,	Prostate	,,	0	,,	Hodgkin's disease	Radiotherapy Chemotherapy	Followed—7 years.
70	F.	Vulva	Sq. cell ca.	0	Excised	Reticulosarcoma	Radiotherapy	Preceded—10 years.
77	M.	Tongue	,, ,, ,,	0	Radiotherapy	,,	,,	Followed—2 years.
65	,,	Colon	Adenocarcinoma	+	Excised	,,	,,	Preceded—9 months.
71	F.	Breast	,,	0	,,	,,	,,	Preceded—8 years.
70	M.	Prostate	,,		None	Lymphosarcoma	None	Simultaneous.

bias in the selection of patients would tend to favour a high incidence of malignant conditions in the patients coming to autopsy. In spite of this the incidence of carcinoma in the patients dying from leukaemia, multiple myeloma, and malignant lymphoma is low. The high incidence of carcinoma in patients dying from malignant disorders of plasma cells noted by Weitzel (1958) was not found in this series, and no special association was found between the development of carcinoma and any other particular type of leukaemia or malignant lymphoma.

Of the 280 patients with a malignant lymphoma, only one had a possible origin in the gastro-intestinal tract. The low incidence of carcinoma in this series (2·1 per cent) compared with the much higher incidence of carcinoma (12·5 per cent) in the series of 40 cases of primary malignant lymphoma of the small and large intestines (Cornes, 1961) remains an interesting observation. These series are too small for any definite conclusions to be made, and further studies are required to determine if there is any significance in the association between carcinoma and lymphoma of the intestinal tract. From the clinical point of view, however, it is important to recognise that carcinomas can develop in these cases, and one should not be too ready in assuming that a second tumour is necessarily a recurrence or a metastasis from the original primary lymphoma.

SUMMARY

1. A survey was made of 14,944 post-mortem reports, and 585 cases of patients dying from leukaemia, malignant disorders of plasma cells, and malignant lymphoma, were selected for study.

2. Sixteen carcinomas were found in this series of 585 cases, an overall incidence of 2·7 per cent. No significant association was found between the development of carcinoma and the different types of leukaemia or malignant lymphoma, and in particular there was no special association between the development of carcinoma and malignant disorders of plasma cells.

3. The low incidence of carcinoma (2·1 per cent) in patients dying from malignant lymphoma is contrasted with a high incidence of carcinoma (12·5 per cent) in patients with primary malignant lymphoma of the small or large intestine. Further studies from other large centres are required to assess the significance of this association.

Appreciation is expressed to Professor C. V. Harrison and to Dr. A. D. Morgan for the opportunity to study post-mortem reports and autopsy material at the Postgraduate and Westminster Medical Schools of London University respectively, and to Dr. I. M. P. Dawson and Dr. B. C. Morson for help with the study of the intestinal tumours. We are grateful to Dr. I. M. P. Dawson, Dr. Cuthbert Dukes and Dr. J. G. Humble, for much helpful advice and criticism. The work was supported by a grant from the British Empire Cancer Campaign.

REFERENCES

BERESFORD, O. D.—(1952) *Brit. J. Cancer*, **6**, 339.
CORNES, J. S.—(1960) *J. clin. Path.*, **13**, 483.—(1961) *Brit. med. J.*, i, 638.
Idem, WALLACE, M. H. AND MORSON, B. C.—(1961) *J. Path. Bact.*, in the press.
HARRISON, C. V.—(1960) 'Recent Advances in Pathology', 7th ed. London (Churchill), p. 44.

MIDER, G. B., SCHILLING, J. A., DONOVAN, J. C. AND RENDALL, E. S.—(1952) *Cancer*, **5**, 1104.
MOERTEL, C. G. AND HAGEDORN, A. B.—(1957) *Blood*, **12**, 788.
MORRISON, M., FELDMAN, F. AND SAMWICK, A. A.—(1944) *Ann. intern. Med.*, **20**, 75.
SLAUGHTER, D. P.—(1944) *Int. Abstr. Surg.*, **79**, 89.
VIDEBAEK, A.—(1947) 'Heredity in Human Leukaemia and Its Relation to Cancer'. London (H. K. Lewis).
WARREN, S. AND EHRENREICH, T.—(1944) *Cancer Res.*, **4**, 554.
WEITZEL, R. A.—(1958) *Cancer*, **11**, 546.

MAIN HISTOLOGICAL TYPES OF PRIMARY EPITHELIAL LUNG TUMOURS

LEIV KREYBERG

From the Institutt for Generell og Eksperimentell Patologi, Universitetet i Oslo, and WHO International Reference Centre for Lung Tumours

Received for publication February 11, 1961

USUALLY lung cancer morbidity and mortality statistics refer to cases registered under No. 162 and 163 in the International List.

Already in 1937, in a discussion in the Medical Society in Oslo, I emphasized the very heterogenous tumour material embraced by the term "lung cancer" and stressed the necessity of distinguishing between the different histological types, when aetiological factors are being studied, as presumably the different histological types also represent biologically different tumour entities. In addition, always looms the uncertainty as to the tumour in question being a primary lung tumour or not.

Not until after the war came the opportunity to study this question systematically. At this time also the manifestation of a new lung cancer situation could be perceived, adding to the pertinency of such studies. The new situation is characterized by: a considerable and progressive increase in lung cancer cases especially in males in urban areas, and a much lower increase in males in rural areas and in women.

More than ten years ago I started collecting as many lung cancer cases as possible in Norway. A minor part were autopsy cases, the major part surgical, including considerable biopsy material.

From the very beginning of my effort to classify the pulmonary epithelial tumours according to histological types, I met methodological difficulties on one hand, and a good deal of scepticism from many colleagues on the other, both natural obstacles, intimately related.

In a series of papers (Kreyberg, 1937, 1954, 1956 and 1959) it has been claimed and shown that a separation of the histological types is not only fruitful, but essential in the study of "lung cancer".

In order to conduct and to promote comparative studies of materials from different sources, it is very important that the different students use identical criteria and nomenclature.

It may therefore be useful to state in some detail the criteria and the nomenclature used by the author in recent years, essentially those laid down by a World Health Organization group of experts meeting in Oslo in 1958, consisting of Professors Dr. J. Delarue, Paris; Dr. H. Hamperl, Bonn; Dr. A. A. Liebow, New Haven; Dr. R. W. Scarff, London; Dr. E. Uehlinger, Zürich; and Dr. L. Kreyberg, Oslo.

A few general remarks may be useful.

Most malignant tumours may occur in more or less differentiated forms, usually without this influencing the nomenclature, even if it may influence the

clinician as to the best treatment and as to prognosis. A few examples may be mentioned.

A malignant melanoma may show a great variety of cells: spindle shaped, globoid or gigantic, and large areas may be without melanin. If, however, an area with melanin formation is identified, the diagnosis is: a malignant melanoma, with a possible addition, " little differentiated " or " highly polymorphous " or similar. No pathologist will diagnose " mixed melanoma and spindle cell sarcoma ", or " mixed melanoma and giant cell sarcoma ".

Another example. The nephroblastic embryonal tumours may in large areas appear with only spindle cells and possibly in a small area only show the characteristic glomerulus-Anlage pattern, giving the clue to the true diagnosis. The smallness of the special area does not invalidate the diagnosis, or make the specific diagnosis subsidiary to something else.

These examples should be kept in mind when we come to the typing of lung tumours, where many conventional pathologists tend to create mixed forms of different types, from the very findings of anaplastic or undifferentiated areas, or structures mimicing specific differentiations.

If we study a series of average primary so-called lung carcinomas we admittedly find a great variance in the size, the form and the differentiation of cells, even within one and the same tumour. Some cells are small, others may be middle sized, others again large. Some cells may be round, others polyhedral or columnar, others again more or less oval to spindle shaped and some may even be giant cells. Accordingly it is possible to apply a number of purely cyto-morphological designations: " small round cell ", " large round cell ", " fusiform ", " giganto-cellular "-carcinomas, etc.

If we come to cyto-functional differentiation, we may find some cells producing mucus, others not, and mucus of different kind, if finer methods are employed for identification. By some stains, as for instance mucicarmine, we actually identify a smaller number of mucus-producing cells than by the use of the alcian stains. A technical warning must be sounded. Sometimes necrotic nuclei are stained blue or green, and even certain degeneration products of the cytoplasm may take the stain and mimic mucus. Next, mucous cells of the bronchial walls may be infiltrated and embedded in the tumour tissue, more or less completely destroyed, and remnants of the mucus be taken as a product of the tumour cells proper. A strict analysis and a critical judgement is necessary, to avoid incorrect conclusions.

Another important cytological differentiation is keratinization with all stages up to complete keratosis with kerato-hyaline granules. Very often the keratinization is manifested only by a tendency of the cytoplasm to stain with eosine, orange-G, saffron and similar stains, which, however, are not specific. Many degenerating and necrotic cells may, on the other hand, take acid stains and resemble keratotic cells. A decision as to the true significance of the staining can sometimes be very difficult or impossible.

The various cyto-functional differentiations may be used in the choice of designation of the tumour, such as: " mucus producing carcinoma ", " keratinizing carcinoma ", etc.

If we next come to the architectural differentiation we may find: solid sheets, ribbons or garlands, buds and strings: we may find acinar or alveolar structures, even papillomas, but also completely anaplastic pictures. Again

designations may be chosen from the histological pattern, such as : " carcinoma solidum ", " adenocarcinoma ", " carcinoma anaplasticum ", etc.

In the different systems of classification a great many combinations of the characteristics mentioned may be construed. In my own typing I have tentatively chosen the path of simplification. If anywhere in a tumour a definitely specific differentiation is found, however limited, this finding decides the type, undifferentiated areas being disregarded. If two different differentiations are found, the tumour is classified as " combined ".

The following types are used :

Epidermoid carcinoma is the designation for all tumours where any definite stratification or whorl formation or any, even traces of cornification have been found, due regard being paid to technical errors and to possible degenerative changes. The cytoplasmatic differentiation can be very marked or very slight. Very anaplastic tumours occur (Fig. 1).

Small cell anaplastic carcinomas may be composed of small polyhedral or of oval cells, usually with very scanty cytoplasm, in smears often with " naked " nuclei, " oat cells " (Fig. 2). The cells may appear in school, ribbons and garlands In many cases fragments may resemble the pictures found in " carcinoma *in situ* ", in the cervix (Fig. 3), and in others great likeness with very little differentiated squamous cell carcinomas (Fig. 4), the complete absence of keratinization then being the essential formal distinction.

In this group usually are also included small cell carcinomas with a growth of small polyhedral to slightly columnar cells, with formation of rosettes and even small lumina, sometimes containing a secretion resembling mucus (Fig. 5). It is possible that these tumours actually represent a special type, which ought to be separated from the other small cell carcinomas. This problem is worth a closer study.

From the age and sex occurrence and the finding of transitional forms it is probable that the small cell tumours actually are undifferentiated epidermoid carcinomas (Fig. 3 and 4).

Adenocarcinoma is the next large and important type. Here the basic morphologic criterium is gland-structures. If such structures are found in any part of the tumour, the base for diagnosis is established, even if large parts are anaplastic, large celled, giant celled or with the cells growing in solid heaps as a " carcinoma solidum " mimicking an epidermoid carcinoma (Fig. 6). Warning must be given that the epidermoid carcinomas, on the other hand, may mimic gland structures, when the cells of the periphery invade alveoles. Cytoplasmatic differentiation, in one or another part of the tumour, with mucus formation, or keratinization may guide to a safe conclusion as to type. A great variety of adenocarcinomas

EXPLANATION OF PLATES.

FIG. 1.—Epidermoid carcinoma with scattered keratinizing cells and no organized architecture.

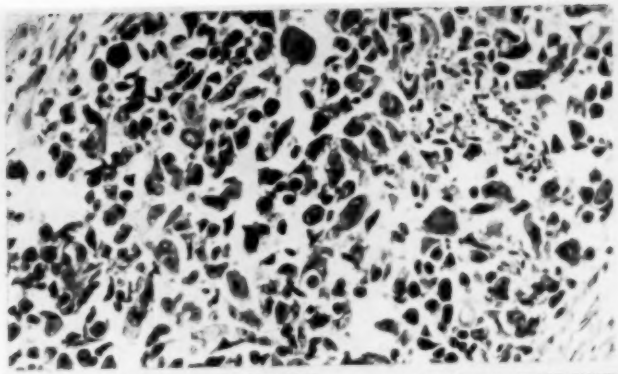
FIG. 2.—" Oat cell " carcinoma with a uniform population of small oval cells.

FIG. 3.—Small cell anaplastic carcinoma with garland formation, no stratification, no trace of keratinization.

FIG. 4.—Small cell carcinoma ? with uncertain stratification and no keratinization—Undifferentiated epidermoid carcinoma ?

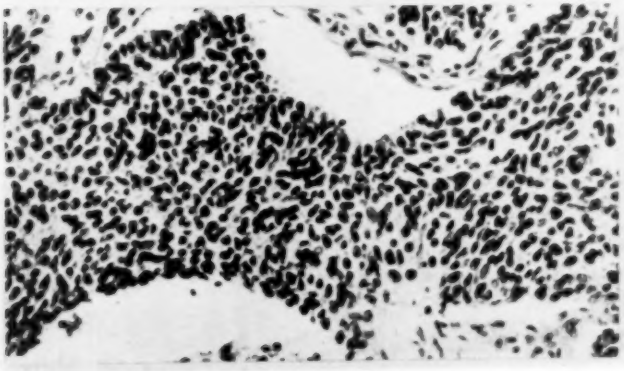
FIG. 5.—Small cell carcinoma with " rosette "—or " gland " like structures. No mucine formation.

FIG. 6.—Undifferentiated area in an adenocarcinoma (Fig. 7 and 8 ; Kreyberg, 1959).

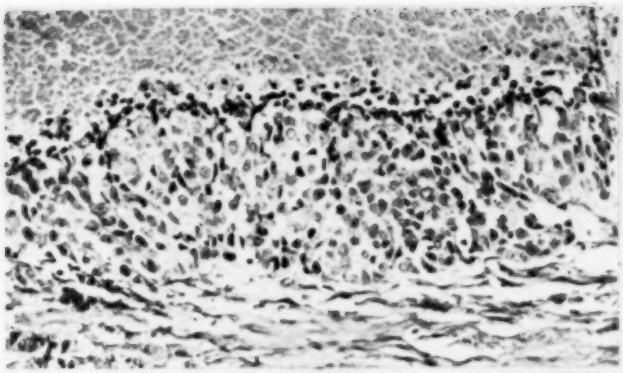


1

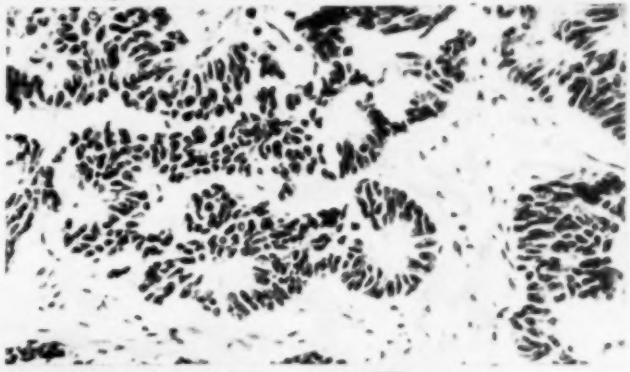
2



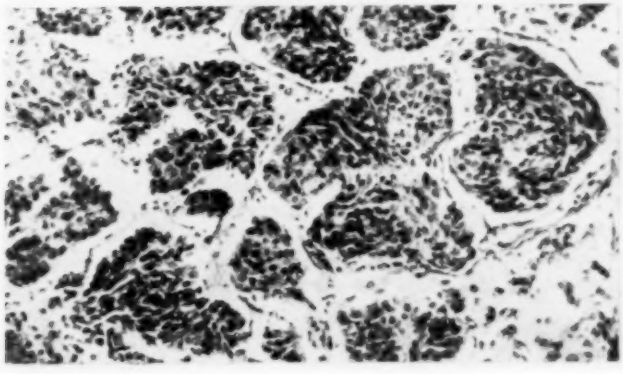
3



4



5



6

are found, including the tumours by Nash and Stout (1958) designated " giant cell carcinomas of the lung ".

If these criteria described, are strictly observed, combined epidermoid adenocarcinomas are found in human material, but rather rare—a few per cent of all, or less.

The bronchiolo-alveolar carcinomas have the well-known characteristic pattern. Certain difficulties may be encountered when being confronted with ovarian lung metastases and some plain primary papillary adenocarcinomas of the lung. This problem is, however, of minor quantitative importance.

Mucous gland tumours, mainly cylindromas, are limited to trachea and the larger bronchii, and these tumours are usually easily identified.

The carcinoids or *adenomas* are, likewise, in most cases easily recognized. Sometimes, however, when deeply infiltrating, a differential diagnosis versus the small cell anaplastic carcinomas must be remembered.

Undifferentiated carcinomas means tumours so undifferentiated that no clue is found permitting them to be included in any of the above types.

From the purely morphological point of view, how many of a total of unselected lung cancers can now be identified according to the criteria mentioned ?

The best answer is not obtained from my own Norwegian material, because that was my first, and I did not keep a precise record of the number of cases discarded because of technical defects, and my own inability to come to a safe conclusion.

A good answer is, however, given by a Finnish material of 624 cases, studied and typed during April–July, 1960. The material, which will be reported upon later, was supplied by a series of Finnish laboratories through the co-operation of Professor Saxén. As " lung cancer " unspecified were recognized all cases where the histological material permitted a diagnosis of " carcinoma ". This was done in order to be in agreement with the clinicians and the statisticians. Some of the cases, could, however, not be used safely for typing, because of defects of the material, in quality or in quantity. A number of cases of good material, however, still left us in uncertainty as to type—undifferentiated carcinoma.

In this ordinary material of routine preparations some 10 per cent were discarded as unfit for typing, and of the remaining bteween 96 and 97 per cent could be typed and classified according to the criteria stipulated. Combined epidermoid-adenocarcinoma was diagnosed in 3 cases only.

I estimate that a similar situation has existed during my study of the Norwegian material.

During the some twelve years where the author has been occupied with attempts at a fruitful typing, the criteria have varied slightly, and even at the present stage it is evident that the final word has not been written on this subject. The classification and the criteria described in the present paper have, however, now reached a stage where the usefulness of the typing is evident, as will be shown in other papers to follow.

SUMMARY

A group of experts convoked by the World Health Organization in Oslo, 1958 proposed a tentative typing of primary epithelial lung tumours.

The criteria have been tested at the International Reference Centre for Lung

Tumours for two years, and the present paper reports on the usefulness of the criteria, add a few details and discuss technical difficulties in interpretation and evaluation.

REFERENCES

KREYBERG, L.—(1937) *Norsk Mag. Lægevidensk.*, **98**. Suppl. Forh. i Det Norske Medicinske Selskab, 36.
Idem.—(1954) *Brit. J. Cancer*, **8**, 199.
Idem.—(1956) *Brit. J. prev. soc. Med.*, **10**, 145.
Idem.—(1959) *Acta Un. int. Cancr.*, **15**, 78.
NASH, A. D. AND STOUT, A. P.—(1958) *Cancer*, **11**, 369.
WORLD HEALTH ORGANIZATION.—(1960) *Epidemiology of Cancer of the Lung. Tech. Rep. Wld Hlth Org.*, No. 192, Genève.

APPENDIX

COMBINED STAINING OF KERATIN AND MUCUS

(Haemalum-Erythrosin-Saffron (Masson) and Alcian Green)

Bring sections to water.
Stain nuclei with Mayer's haemalum.
Blue in water.
Stain with erythrocin (1 per cent aqueous sol.), 3 min.
Rinse in water.
Differentiate quickly in 80 per cent alcohol.
Stain with Alcian Green, 5 min.
Rinse in water.
Cover sections with Saffron solution.
Drain, blot with filter paper.
Dehydrate in 3 changes of absolute alcohol.
Clear and mount in Canada balsam.

Alcian Green :

Alcian Green (1 per cent aqueous solution)	50 c.c.
Acetic acid (1 per cent)	50 c.c.
Thymol	20 mg.

Filter before use.

Saffron :

Saffron (dried stamens)	6 g.
Distilled water	300 c.c.

Boil 1–2 hours in a water bath.
Cool, filter and add :

Tannic acid (5 per cent)	3 c.c.
Formol (40 per cent)	3 c.c.

(WHO International Centre for Lung Tumours, Rikshospitalet, Oslo.)

A COMPARISON OF LUNG TUMOUR TYPES IN FINLAND AND NORWAY

LEIV KREYBERG AND ERKKI SAXÉN

From the Institutt for Generell og Eksperimentell Patologi, Universitetet i Oslo, Norway, and Universitetets Patologisk-anatomiska Avdeling, Maria Sjukhus, Helsingfors, Finland

Received for publication March 22, 1961

If the thesis, that the main increase in lung cancer in large areas of the world is caused by epidermoid and small cell anaplastic carcinomas (Group I tumours), and that the other types: adenocarcinomas, carcinoids, mucous gland tumours and possibly also bronchiolo-alveolar cell carcinomas (Group II tumours) remain fairly stationary, the ratio Group I : Group II tumours should be a rough, but useful indicator of interest for epidemiological and aetiological studies (Kreyberg, 1959).

In addition to the material given at the presentation of this thesis further studies have been undertaken.

Kreyberg (1961*a*) has, in a Norwegian material of 522 cases of lung cancer in males, shown a linear relationship between the ratio mentioned and the amount of tobacco smoked. Only at the lowest and highest smoking levels (up to 4 and above 29 g.) the graph deviated from the straight line. The findings are in full agreement with an earlier investigation of a British material (Doll, Hill and Kreyberg, 1957). The similar results were, however, obtained through the use of different methods. The British material was examined as to the *rate* occurrence and the Norwegian as to the *ratio* findings.

The thesis has furthermore been tested on a very small Italian material (78 cases only) from the city of Venice (Ferrari and Kreyberg, 1960).

Those findings, at a first glance, did not confirm the thesis. Other circumstantial evidence pointing in the direction of a high frequency of lung cancer in Venice was contrasted by a ratio Group I : Group II tumours of 3·2 : 1 in males. The corresponding ratio for 522 Norwegian males was 3·4 : 1, and the frequency of lung cancer in Norway is only 12–13 per 100,000 males (Dr. Pedersen, The Norwegian Cancer Registry).

A further study of the two materials, however, disclosed some remarkable differences in their composition. In the Italian material 67 out of 78 cases were autopsy cases, whereas in the Norwegian material the figures were 36 out of 600.

The biopsy and autopsy cases in males examined separately give figures as shown in Table I.

TABLE I.—*Group I : Group II.* Males.

	Biopsies		Autopsies	
	Ratio	Number	Ratio	Number
Norway	3·6 : 1	488	1·8 : 1	34
Venice	10 :	10	2·5 : 1	49

Considerable differences were also observed as to the age of the lung cancer patients in the two materials.

These different findings strongly underline the necessity, not only of adhering to identical histological criteria, but also of paying attention to the composition of the materials to be compared.

The next project in our systematic studies, encouraged by the World Health Organization (1960), was an examination of the condition in Finland. This country offers the especially interesting combination of a very high lung cancer incidence and low industrialization.

Professor Saxén obtained the generous co-operation of Professors O. Järvi, V. Ritama, K. Setälä, H. Teir, and U. Uotila, and this resulted in histological material from 624 unselected cases of "lung cancer", mainly from the years 1957–59.

Blind-typing, that is typing with no information regarding sex, age, previous diagnosis and similar, was carried out by Kreyberg. The diagnoses were accepted by Saxén, and the result is given in Table II.

TABLE II.—*Lung Cancer Types.*

Unselected material, Finland 1958–59 (Saxén *et al.*)

	Males		Females		
Group I carcinomas:					
Epidermoid carcinomas	333		2		
Small cell anaplastic carcinomas	152	485	5	7	
Group II carcinomas:					
Adenocarcinomas	38		13		
Carcinoids	2	40	2	15	
Combined epidermoid-adenocarcinomas		3			
Uncertain interpretation		19		1	
Necrotic or too small for typing		50		4	
Total		597		27	624

The histological criteria used are those laid down in a recent paper (Kreyberg, 1961*b*), actually the same as used in the British, Italian and Norwegian materials.

As "lung cancer" unspecified were recognized all cases where the histological material permitted a diagnosis of carcinoma. This was done in order to be in agreement with the clinicians and the statisticians. Some of the cases, however, could not be used safely for typing, because of defects in quality or quantity of the material, "necrotic or too small". A number of cases with good material, however, still left us in uncertainty as to type, listed under "uncertain interpretation".

In this ordinary material of routine preparations, however, less than 10 per cent were discarded as unfit for typing, and of the remaining, approximately 97 per cent could be typed and classified according to the criteria stipulated.

Remarkable is the very low number of combined epidermoid-adenocarcinomas, 3 cases only. This material will be examined more extensively in a later paper. In the present, only a few features will be studied.

The ratio Group I : Group II tumours in males in the total material is very high, 12·1 : 1. And again, if the biopsy and the autopsy cases are examined separately, the same remarkable difference is found, as previously observed in

TABLE III.—*Ratio Group I : Group II.*

Males, Finland.

	Ratio	Number of cases
Total material	12·1 : 1	485 : 40
Biopsies	18·1 : 1	427 : 23
Autopsies	3·4 : 1	58 : 17

Norway and Italy. Admittedly, the autopsy cases are not numerous in any of these materials, but the findings are so consistent, that the conclusion most

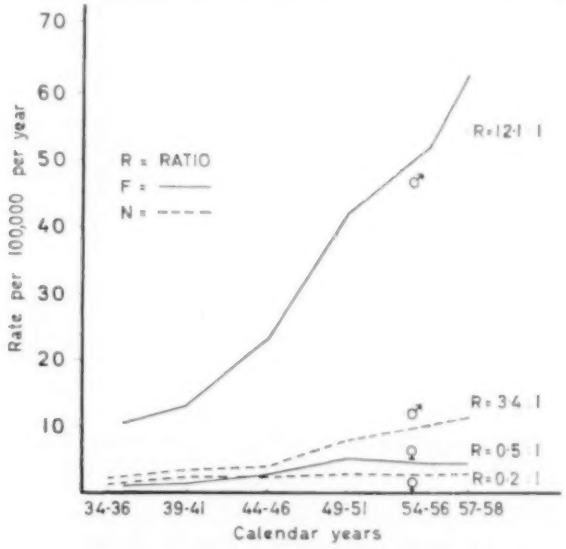


FIG. 1.—'Lung cancer'. Finland–Norway.

probably is correct, that as groups the lung cancer patients being biopsied differ essentially from those being autopsied. This is a very important methodological point, when comparative studies are carried out. Of interest will be a report of a large material of autopsy cases typed according to the criteria used in our studies.

The ratio Group I : Group II tumours in females is only useful in the total material, as the number of cases is too low to be subdivided. The ratio is 0·5 : 1 (22 cases).

The main point to be examined in the present paper, is the relationship between the lung cancer rates and ratios in Finland and Norway.

It shall be stressed that as regards *rates*, under "lung cancer" the figures comprise the No. 162 and 163 of the International List.

The *ratios*, on the other hand, presumably contain nearly only No. 162 cases, as considerable care has been exerted to exclude secondary lung tumours. This difference in composition may be raised as a theoretical objection, but the error is presumably of a similar order of magnitude in the two countries.

That the Finnish and the Norwegian materials are fairly similar also as regards sources is shown by the fact that in males the Finnish shows 14·3 per cent autopsy cases and the Norwegian 6·9 per cent.

Fig. 1 illustrates the enormous differences in lung cancer *rate* in males in the two countries as well as the much lower rates for females, with a small overweight for Finnish women. Next, the graph shows correspondingly large differences not only in the *ratios* in males, but also a complete agreement for the females with their much lower but consistent differences.

If, on the basis of these findings, the results of the Italian study are reexamined, it may be that the initial impression of a discrepancy between the thesis advanced and the ratio found in Venice is open for a revision. In the Venice male biopsy series 10 Group I tumours are standing against no Group II tumours, and the autopsy ratio in males of 2·5 : 1 is intermediate between the very low Norwegian of 1·8 : 1 and the higher Finnish of 3·4 : 1. This would, according to the thesis, bring the Venice rate somewhere between the acknowledged very high Finnish and very low Norwegian rates, which would be in good accord with other information regarding the lung cancer situation in Venice, and Italy in general.

SUMMARY

The thesis, that the main increase in lung cancer in large areas of the world is caused by epidermoid and small cell anaplastic carcinomas (Group I tumours), whereas the other types, mainly adenocarcinomas (Group II tumours), remain fairly stationary, and that therefore the ratio Group I : Group II tumours gives a rough indicator useful for epidemiological work on lung cancer has been studied by a comparison of two large materials, one from Finland and one from Norway. Methodological questions are discussed, partly with reference to a small material from Venice, and the findings illustrated in Fig. 1 strongly support the thesis advanced.

The findings further underline the importance of uniformity of criteria and of source and composition of materials for comparative studies.

REFERENCES

Doll, R., Hill, A. B. and Kreyberg, L.—(1957) *Brit. J. Cancer*, **11**, 43.
Ferrari, E. and Kreyberg, L.—(1960) *Brit. J. Cancer*, **14**, 609.
Kreyberg, L.—(1959) *Acta Un. int. Cancr.*, **15**, 78.—(1961*a*) *Brit. J. Cancer*, **15**, 51.—(1961*b*) *Ibid.*, **15**, 206.
World Health Organization.—(1960) *Tech. Rep. Wld Hlth Org.* No. 192.

CHANGES IN THE BRONCHIAL EPITHELIUM IN PRIMARY LUNG CANCER

R. CARROLL

*From the Department of Pathology, Postgraduate Medical School of London**

Received for publication February 24, 1961

THE association of squamous metaplasia of the bronchial epithelium with carcinoma of the lung has previously been noted by Tuttle and Womack (1934), Lynch and Smith (1934), Mulligan and Harper (1943) and Niskanen (1949). Isolated cases of intraepithelial carcinoma (epidermoid carcinoma *in situ*) of the bronchial epithelium have been described by Gray and Cordonnier (1929), Reingold, Ottoman and Konwaler (1950) and Papanicolaou and Koprowska (1951) but the relatively frequent association of this finding with frankly invasive lung cancer was not stressed till Black and Ackerman (1952) stated that epidermoid and undifferentiated tumours of the lung arose first as intraepithelial carcinomas. The position of basal cell hyperplasia is less clear and this study was undertaken in an attempt to clarify the relationship of squamous metaplasia, basal cell hyperplasia and intraepithelial carcinoma of the bronchial epithelium with invasive primary lung cancer.

MATERIAL AND METHODS

The material consisted of 92 primary lung tumours resected at Hammersmith Hospital between 1952 and 1958. Only those tumours in which there was bronchial epithelium in the sections are included in this group. The site of origin of each tumour was determined as accurately as possible. The bronchi of many of the specimens were distended by formalin (15 per cent formol saline) and were allowed to fix overnight before dissection was carried out.

The number of blocks taken for section depended on the size of the tumour and varied from 2 to 8. All the sections were stained with Haemalum and Eosin (H. & E.) and selected ones were stained with Periodic Acid-Schiff (PAS) technique, Alcian Green, Feulgen reaction for deoxyribonucleic acid (DNA), Best's stain for glycogen and the Oil Red stain (Pearse, 1953).

The tumours were classified histologically and the bronchial epithelium in the sections was carefully studied. In every tumour examined the bronchial epithelium adjacent to the tumour was present and in many cases bronchial epithelium at some distance from the tumour was also present.

Histology

Three main histological types of tumour were recognised. Fifty-three (58 per cent) were classified as squamous carcinoma, 14 (15 per cent) as oat cell carcinoma and 11 (12 per cent) as adenocarcinoma. Fourteen (15 per cent) were found to be unclassifiable. The large number of unclassifiable tumours was due to the fact

* Present address: Department of Pathology, University of Liverpool.

that only those tumours in which complete agreement as to the histology was reached by a number of observers were classified as shown. This was thought to be essential as an attempt was being made to correlate changes in the bronchial mucosa with histological type. The unclassified group largely showed no tendency to differentiation.

Changes in the bronchial epithelium

Basal cell hyperplasia was deemed to be present if there were three or more layers of basal cells. Basal cells, usually one layer thick, form the deepest row of cells in the bronchial epithelium and are situated just above the basement membrane. Their nuclei are frequently round but sometimes elongated and parallel to the basement membrane. Metaplastic squamous epithelium of the bronchi resembles squamous epithelium in other parts of the body ; it forms intercellular bridges but only rarely shows keratinisation. In those of the present series of cases in which intraepithelial carcinoma was present in the lining epithelium, the basement membrane was intact. The epithelium was thickened and there was cellular disorganisation with loss of the usual layering. The nuclei showed a great variation in size, shape, and chromatin content, usually with hyperchromatism. Mitotic figures were numerous and the nuclear cytoplasmic ratio was altered in favour of the former.

TABLE I.—*Relation between Histological Type and Changes in the Bronchial Epithelium*

Histology of tumour	Total number of each histological type	Basal cell hyperplasia	Squamous metaplasia	Intra-epithelial carcinoma
Squamous carcinoma	53	27	12	14
Oat cell carcinoma	14	7	1	0
Adenocarcinoma	11	0	1	0
Unclassified	14	6	1	1

Cases which showed squamous metaplasia and intraepithelial carcinoma or basal cell hyperplasia and intraepithelial carcinoma were included under both headings. Similarly cases which showed basal cell hyperplasia and squamous metaplasia were also included under both.

In the squamous carcinoma group 8 cases showed both basal cell hyperplasia and intraepithelial carcinoma, whereas only 1 case showed both squamous metaplasia and intraepithelial carcinoma. Three cases showed both squamous metaplasia and basal cell hyperplasia. Apart from the figures noted above for oat cell carcinoma there were 2 cases in which the lining epithelium of the bronchi was replaced by spindle shaped cells but it was difficult to decide whether this was due to a proliferation of intermediate cells (these cells form the second layer of the bronchial epithelium, they are spindle shaped and extend from the basement membrane to the surface and may end in a point or in a rounded extremity) confined to the normal thickness of the epithelium or whether it was due to invasion of the bronchial epithelium by tumour. Cases of the adenocarcinoma type showed very little change in the bronchial epithelium and this probably coincided with the fact that 9 of these tumours had a peripheral origin and only 2 had a central origin. The site of origin of the tumours was determined according to the

criteria laid down by Walter and Pryce (1955). In the unclassified group there was 1 case which showed both basal cell hyperplasia and squamous metaplasia.

DISCUSSION

The two most significant facts from Table I are firstly, the high incidence of basal cell hyperplasia in the squamous, oat cell and unclassified groups and secondly the marked changes in the bronchial epithelium in squamous carcinoma.

The first fact appears to substantiate Kreyberg's (1954) hypothesis that squamous cell carcinoma and large and small cell carcinoma are a distinct biological entity from adenocarcinoma and other related types of lung tumour. It also gives some support to Patten, McDonald and Moersch's (1951) theory that the prognosis of a group of tumours which show no tendency to differentiation may be more closely related to that of the squamous carcinoma type than any other type, in other words, that tumours which show no differentiation are probably high grade squamous carcinomas and carry some of the relatively good prognosis of this group.

With respect to the second fact, Auerbach *et al.* (1957*a*) in a series of 54 cases of early invasive cancer of the lung, which included 33 squamous carcinomas and 16 undifferentiated carcinomas, found that 48 showed evidence of intraepithelial cancer of the bronchial epithelium. Auerbach *et al.* (1957*b*) found that the application of a carcinogenic agent to the bronchial epithelium gave rise to a number of changes, i.e. hyperplasia, metaplasia and intraepithelial cancer which usually preceded the occurrence of invasive cancer and a study of the bronchial epithelium in patients who died of carcinoma of the lung showed these changes to be widespread. An investigation by Hamilton *et al.* (1957) showed that basal cell hyperplasia was the commonest change in the lining epithelium of the bronchi in both smokers and in cases of cancer of the lung and Auerbach *et al.* (1956) have shown that basal cell hyperplasia is much commoner in smokers than non-smokers, whereas squamous metaplasia is only slightly commoner in the smoker. Black and Ackerman (1952) reviewed 60 cases of undifferentiated and epidermoid carcinoma of the lung and found intraepithelial carcinoma in 22 per cent and basal cell hyperplasia in 13 per cent of cases. The relationship between basal cell hyperplasia, intraepithelial carcinoma and invasive squamous carcinoma is well established for the cervix (Gusberg and Moore, 1953 ; Carson and Gall, 1954), and the same process of development appears to be true for the bronchial epithelium.

Niskanen (1949) considered that metaplasia arose through regenerative activity of the basal cells. The regenerating cells as they proliferate adopt more and more the morphological characteristics of squamous cells and the most advanced degree of differentiation is represented by hornified squamous epithelium. This is analogous to the process which occurs in the oesophagus in embryonic life when spindle cells with hyperchromatic nuclei proliferate and spread upwards to replace the ciliated and goblet cells of the mucosa, at the same time gradually acquiring squamous features. Niskanen (1949) found squamous metaplasia in 50 per cent of cases of chronic pulmonary diseases and also a tendency to squamous metaplasia seemed equally common to both sexes. He concluded that the incidence of squamous carcinoma was no more frequent in the squamous metaplasia group than in the general population and that therefore squamous metaplasia should not be regarded as a precancerous condition.

There appears to be no doubt about the fact that squamous carcinoma arises

from the epithelium lining the main bronchi both from the associated changes in the bronchial epithelium and the position of the tumour (83 per cent had a central origin in this series). The association of squamous metaplasia and squamous carcinoma is probably coincidental but basal cell hyperplasia and intraepithelial carcinoma of the bronchial epithelium are stages in the development of invasive squamous carcinoma. The power of the basal cells to form fully hornified squamous epithelium is seen in many conditions, especially bronchiectasis and pulmonary tuberculosis and it is logical to suggest that a carcinogen may cause an unchecked proliferation of these cells and result in a fully developed squamous carcinoma. If we are to regard squamous metaplasia as a precancerous condition we should expect a higher incidence of squamous carcinoma in women and a higher incidence of squamous carcinoma in conditions which give rise to squamous metaplasia of the bronchial epithelium.

Smoking is regarded as playing a large part in the aetiology of lung cancer (Doll and Hill, 1952 and 1954) but it has been shown that basal cell hyperplasia is commoner than squamous metaplasia in the bronchial epithelium of smokers (Auerbach *et al.*, 1956; Hamilton *et al.*, 1957).

Weller (1953) cast some doubt on the fact that epithelial metaplasia of the bronchial epithelium might precede pulmonary neoplasia and in a comprehensive study of a large number of unselected autopsies came to the conclusion that the epithelial response might be secondary to the primary disease. His dormant type of transitional metaplasia is identical with the basal cell hyperplasia described in this paper.

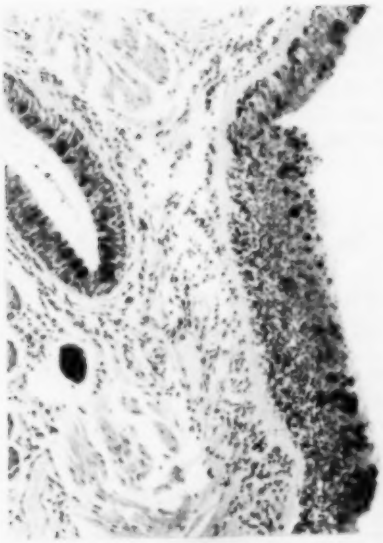
Basal cell hyperplasia was the commonest finding in the oat cell carcinoma group and in 2 extra cases not included in Table I the cells were quite spindle shaped and resembled closely the intermediate cells of the bronchial epithelium. The cell type of oat cell carcinoma is probably either the basal cell or the intermediate cell of the bronchial epithelium. The predominant cell in the tumour is usually the spindle cell and the fact that a certain amount of differentiation toward tubule formation exists (Azzopardi, 1959) suggests that the cell type may be intermediate cell. The intermediate cells are probably somewhat more differentiated than the basal type. Some of the tumours do not contain tubules and these may be formed entirely of basal cells.

It therefore appears that a large number of primary carcinomas of the lung, (squamous, oat cell and unclassified types), arise from the basal cells of the bronchial epithelium and that the malignant cells may exhibit one or more lines of cytological differentiation or alternatively show no tendency.

Normally the germinative cells in the basal layer of the bronchial epithelium grow out to replace adult cells which have been injured or destroyed. In most instances this occurs without ill effects, and the result is normal epithelium or squamous metaplasia if the process is a chronic one. If, however, a predisposition towards cancer exists in the host, malignant cells may arise from the basal layers and cancer results.

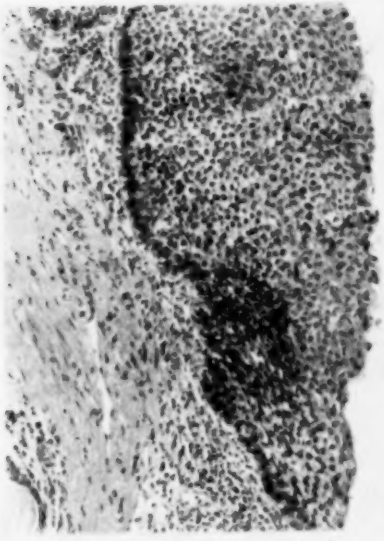
EXPLANATION OF PLATE.

FIG. 1.—Basal cell hyperplasia of the bronchial epithelium. H. and E. ×64.
FIG. 2.—Squamous metaplasia of the bronchial epithelium. H. and E. ×64.
FIG. 3.—Intraepithelial carcinoma of the bronchial epithelium. H. and E. ×87.
FIG. 4.—Spindle shaped cells in the bronchial epithelium of a case of oat cell carcinoma. H. and E. ×64.

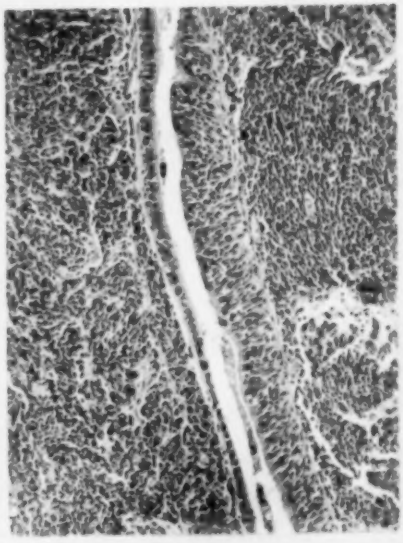


1

2



3



4

SUMMARY

The bronchial epithelium of 92 primary lung cancers was examined. Basal cell hyperplasia was the commonest change found in the epithelium and was confined to the squamous, oat cell and unclassified groups. The bronchial epithelium of the squamous carcinoma group showed the most extensive changes. Approximately 50 per cent of the cases of squamous carcinoma showed basal cell hyperplasia and a significant number showed squamous metaplasia and intraepithelial carcinoma.

The relevant literature is discussed and the association of squamous carcinoma and squamous metaplasia is regarded as coincidental whereas basal cell hyperplasia and intraepithelial carcinoma represent stages in the development of squamous carcinoma and possibly also oat cell carcinoma and the undifferentiated types of carcinoma of the lung.

I wish to acknowledge the interest and advice of Professor C. V. Harrison and Dr. B. E. Heard. I wish to thank Mr. W. H. Brackenbury for the photographs.

REFERENCES

Auerbach, O., Petrick, T. G., Stout, A. P., Statsinger, A. L., Muehsam, G. E., Forman, J. B. and Gere, J. B.—(1956) *Cancer*, **9**, 76.

Idem, Gere, J. B., Pawlowski, J. M., Meuhsam, G. E., Smolin, H. J. and Stout, A. P.—(1957*a*) *J. thorac. Surg.*, **34**, 298.

Idem, Gere, J. B., Forman, J. B., Petrick, T. G., Smolin, H. J. ,Muehsam, G. E., Kassouny, D. Y. and Stout, A. P.—(1957*b*) *New Engl. J. Med.*, **256**, 97.

Azzopardi, J. G.—(1959) *J. Path. Bact.*, **78**, 513.

Black, H. and Ackerman, L. V.—(1952) *Ann. Surg.*, **136**, 44.

Carson, R. P. and Gall, E. A.—(1954) *Amer. J. Path.*, **30**, 15.

Doll, R. and Hill, A. B.—(1952) *Brit. med. J.*, ii, 1271.—(1954) *Ibid.*, i, 1451.

Gray, S. H. and Cordonnier, J.—(1929) *Arch. Surg.*, **19**, 1618.

Gusberg, S. B. and Moore, D. B.—(1953) *Obstet. Gynec.*, **2**, 1.

Hamilton, J. D., Sepp, A., Brown, T. C. and McDonald, F. W.—(1957) *Canad. med. Ass. J.*, **77**, 177.

Kreyberg, L.—(1954) *Brit. J. Cancer*, **8**, 199.

Lynch, K. M., and Smyth, W. H.—(1934) *Amer. J. Cancer*, **24**, 56.

Mulligan, R. M. and Harper, R. F.— 1943) *J. thorac.-Surg.*, **12**, 734.

Niskanen, K. O.—(1949) *Acta path. microbiol. scand.*, **26**, 1.

Papanicolaou, G. N. and Koprowska, I.—(1951) *Cancer*, **4**, 141.

Patten, M. M., McDonald, J. R. and Moersch, H. J.—(1951) *J. thorac. Surg.*, **22**, 88.

Pearse, A. G. E.—(1953) 'Histochemistry: Theoretical and Applied.' London (Churchill).

Reingold, I. M., Ottoman, R. E. and Konwaler, B. E.—(1950) *Amer. J. clin. Path.*, **20**, 515.

Tuttle, W. M. and Womack, N. A.—(1934) *J. thorac. Surg.*, **4**, 125.

Walter, J. B. and Pryce, D. M.—(1955) *Thorax*, **10**, 117.

Weller, R. W.—(1953) *Amer. J. clin. Path.*, **23**, 768.

BOTRYOID SARCOMA OF THE VAGINA

R. SALM

From the Rivers Pathological Department, Camborne-Redruth Hospital, Redruth, Cornwall

Received for publication February 9, 1961

BOTRYOID sarcoma of the vagina in children is a rare tumour and any one observer may well not encounter a single case during his working life. Thus opinions expressed on the tumour's natural history based on single cases may not reflect the properties of the majority. To this circumstance one must probably attribute statements in even the more recent literature that sarcoma botryoides is a tumour which is mainly locally invasive and rarely metastasizes (Duncan and Fahmy, 1953; Ulfelder and Quan, 1947; Sharp and Helwig, 1959; Taylor, 1958; MacGregor, 1960). It would appear likely that this misconception is due to one of two circumstances. In the first place local spread may cause compression of the ureters resulting in hydronephrosis and death due to uraemia before the growth has had time to produce metastatic deposits. Secondly, the diagnosis is made not infrequently in hospital, but the patient is allowed to return home and as often no autopsy can be arranged, no records are made available as to the ultimate features. On the other hand it must be admitted that some botryoid sarcomas do run a protracted course and metastasize late. Thus Daniel, Koss and Brunschwig (1959) recorded a case (their case 5) in which death occurred 6 years after the appearance of the first symptoms.

This report presents a single case of this distressing tumour type, in which full clinical and necropsy data have been obtained, demonstrating widespread dissemination of growth.

CASE HISTORY

A girl then aged 2 years was taken by her mother to see the family doctor in April 1959, as on occasions some ill-defined structures had been appearing in the introitus vulvae. The physician was unable to detect anything abnormal on external examination and referred the patient to the gynaecologist. He also was unable to find any abnormality on ordinary examination, but repeating his examination under an anaesthetic he noticed several small polypoidal structures high up in the vaginal vault, and these were removed for histological examination. After a diagnosis of botryoid sarcoma had been returned the problem of therapy was considered. After consulting various authorities in this country and abroad it was decided to apply radium intravaginally, and 6000 r were given in 5 days. Ten days after removal of the applicator a large polypoidal structure of irregular shape was expelled from the vagina. Apart from a moderate degree of anaemia with haemoglobin estimations of about 60 per cent the child appeared to be in good health. The parents were fully informed of the position before the patient was discharged and she was nursed, very adequately, at home by her mother. Her abdomen was noted to enlarge gradually, which the family doctor thought to be due to ascites.

Her condition slowly deteriorated and death occurred in November 1959, 7 months after the mother had first become suspicious, and 4 months after the diagnosis had been established in hospital.

Necropsy

This was performed 18 hours after death. The abdomen was grossly distended and tense, with protruding umbilicus. A network of distended subcutaneous veins was discernible on the anterior chest wall, which communicated with distended veins running perpendicularly on either side of the midline across the abdomen. The vulval skin was swollen and oedematous, and soft tumour tissue was palpable just behind the introitus.

On opening the abdominal cavity a large tumour mass presented which proved to be neoplastic omentum, measuring 20 × 15 × 4 cm. and weighing 1110 g. There was no ascites, and obstruction of the intra-abdominal venous drainage, as indicated by the ancillary subcutaneous venous plexuses, must have been due to compression of the large abdominal veins by tumour masses from without, as their lumina were patent throughout. After separating some adhesions with the anterior abdominal wall the tumourous omentum could be turned upward and the entire parietal peritoneum was seen to be covered by a smooth, firm, whitish sheet of tumour tissue, measuring 3–7 mm. in width, which had also spread along the mesentery, was enveloping the large gut, liver and spleen, and was encroaching upon the wall of the small gut, especially the ileum and lower jejunum (Fig. 1). The pelvic organs were removed *en bloc* and bisected sagittally. Altogether they weighed 2750 g. The vagina was grossly distended, measuring 6 cm. in diameter, and filled with grape-like tumour masses (Fig. 2). The tumour was found to have spread into the vaginal wall, the cervix, all but the fundal part of the uterine body, the bladder and both parametria, and had given rise to a large tumour mass behind the uterus which had obliterated the pouch of Douglas. The rectal wall was not involved. The distal part of the left tube and the left ovary were identifiable, the right-sided appendages had been incorporated in the retro-uterine mass. In the bladder the growth, which was still covered by intact epithelium, had formed low polypoidal protuberances, producing a cobblestone appearance. The para-aortic lymph glands were grossly enlarged and coalescing, forming tumorous deposits, measuring up to 10 cm. in length. Further involved lymph glands were found in the anterior mediastinum and in the right infraclavicular region. Multiple button-shaped subpleural deposits were present in both lungs. The 4th left rib, near the vertebral column, had been completely destroyed by a single large metastasis, whilst the body of vertebra S I was diffusely invaded by tumour growth. Both renal pelves and ureters were moderately dilated. The ureters were patent to a probe, though some resistance was encountered in their pelvic segments due to compression by tumour tissue from without. Skull and brain, as well as main abdominal organs, were not involved.

Histology

Vaginal biopsy (59/1555).—The material submitted consisted of a dozen small polypoidal structures, clothed by smooth, glistening epithelium. On microscopical examination they proved to consist of loose, oedematous cellular tissue surrounded by normal stratified epithelium (Fig. 3). The bulky core was made up of small,

undifferentiated cells with small hyperchromatic nuclei. Immediately below the epithelium the cells formed a more compact cellular layer. In one of the polypi more cellular areas were discernible, displaying some large, elongated, strap-like cells with strongly eosinophil cytoplasm, and these, with special stains, exhibited well developed cross-striations (Fig. 4). No intercellular mucin was demonstrable.

Post-mortem material (59/2680).—Nineteen blocks were cut from various sites. The sections from all showed a cellular pleomorphic sarcoma, the cells of which usually lacked any differentiation. Only after prolonged search a few cells were found in sections from the omental mass, which displayed definite cytoplasmic cross-striation (Fig. 5). Mitotic figures were present throughout in moderate numbers. On the hepatic and splenic surfaces the tumour layer was superimposed loosely onto the intact capsules. In the 4th left rib and in S I the neoplasm had grown diffusely, replacing the bone marrow and resulting in partial or complete destruction of bone trabeculae. The tumour displayed a pronounced tendency to form polypoidal masses on the surface of hollow organs, such as bladder, cervix and vagina, whilst the same tendency was noted in preformed microscopical cavities, such as bronchi (Fig. 6), alveoli and lymph gland sinusoids (Fig. 7). In lymph glands and bone marrow multinucleated tumour giant cells were conspicuous.

DISCUSSION

The main macroscopical and microscopical features of the present case are those of botryoid sarcoma of the vagina in children. Uncommon findings were the demonstration of polypoidal ingrowths into preformed microscopical cavities such as bronchi, lung alveoli and sinusoids of lymph glands, the presence of multinucleated tumour giant cells in some of the metastatic deposits, the formation of large intra-abdominal metastatic masses, the marked coelomic spread, and the widespread dissemination via lymphatics and bloodstream. As is usually the case the wall of the gut, and especially the rectal wall, were spared, and the ureters, though compressed from without, were not involved.

Grape-like mesenchymal tumours of fairly similar macroscopical and microscopical appearances, including rhabdomyoblasts, and of high malignancy have

EXPLANATION OF PLATES

FIG. 1.—Opened abdominal cavity with tumorous omentum turned upwards. Smooth tumour layer lining parietal peritoneum, covering fundus of bladder and uterus, and encroaching upon serosal aspect of small gut. About $\times \frac{1}{3}$.

FIG. 2.—Bisected pelvic organs showing thickened bladder and vaginal walls and retro-uterine mass; distended vagina filled with grape-like tumour masses; and polypoidal appearance of bladder lining. About $\times \frac{1}{3}$.

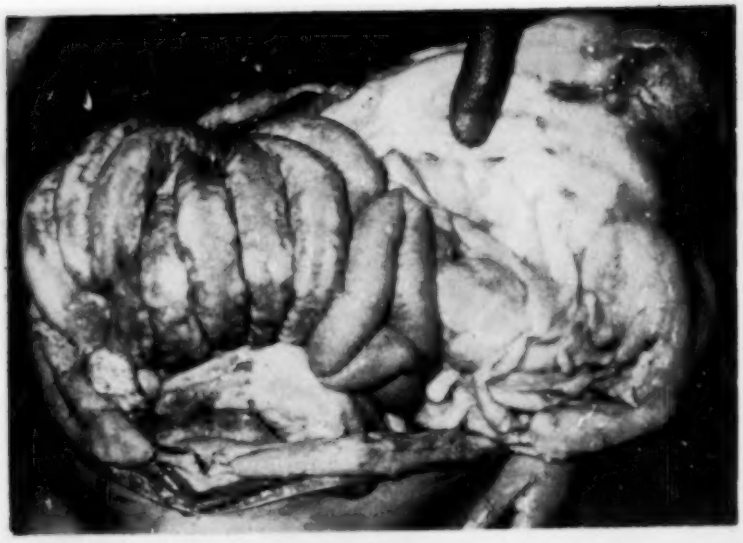
FIG. 3.—Vaginal biopsy showing polypoid tumour clothed by normal stratified epithelium. Cellular cambium layer underneath, followed by loose-textured oedematous tumour tissue. Haematoxylin and eosin. $\times 23$.

FIG. 4.—Vaginal biopsy, high power field with rhabdomyoblast. Phosphotungstic acid and haematoxylin. $\times 473$.

FIG. 5.—Composite picture of tumour cells from omental mass showing cross-striation. Phosphotungstic acid and haematoxylin. $\times 473$.

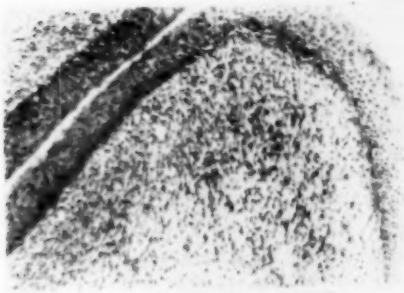
FIG. 6.—Lung metastasis showing polypoidal ingrowth into small bronchus. Bronchial epithelium intact. Haematoxylin and eosin. $\times 26$.

FIG. 7.—Invaded abdominal lymph gland with polypoidal ingrowth into peripheral sinusoid. Haematoxylin and eosin. $\times 23$.

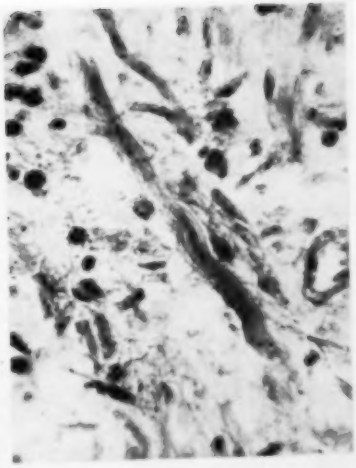


1

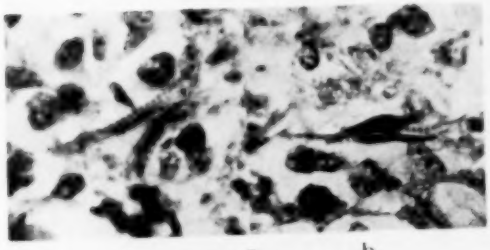
2



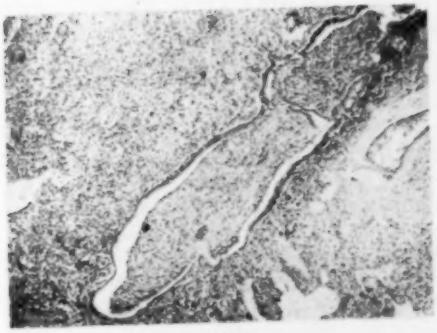
3



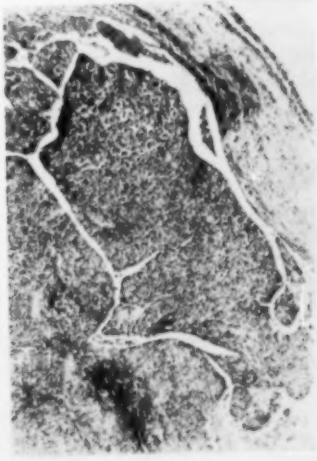
4



a 5 b



6



7

been observed by several investigators as arising at various sites of the head and neck in children and adolescents: in the nasopharynx, soft palate and tonsil; meatus, middle ear and area of Eustachian tube; parotid, orbit and temperozygomatic region (Nicory, 1923; Martin and Alexander, 1924; Söderberg, 1933; Cappell and Montgomery, 1937; Maconie, 1944; Stobbe and Dargeon, 1950; St. John and Wood, 1955; Prior and Stoner, 1957; Horn and Enterline, 1958), and recently identical botryoid sarcomas have been observed to originate in the common bile duct before puberty (Horn, Yakovac, Kaye and Koop, 1955; Farinacci, Fairchild, Sulak and Gilpatrick, 1956), whilst the occurrence of this tumour type in bladder and urethra of both sexes, and in the prostate, though rare, has been known for many years (Eibergen, 1952; Hanbury, 1952; Mostofi and Morse, 1952).

But the great majority of botryoid sarcomas do arise in vagina and cervix, and, less frequently, in the uterine body; in the uterus usually after the age of 20. A single case is on record where the tumour originated in the hymen of a girl aged 3 years (Edwards, Sheboygan and Richardson, 1933).

It is often stated that the grape-like neoplasm of the pelvic area arises in tissues and organs derived from the urogenital sinus. But in view of an interesting report of an anal botryoid sarcoma, mentioned briefly by Ober and Edgcomb (1954) and reported more fully by Sharp and Helwig (1959), it would be more accurate to postulate a derivation from tissues of cloacal origin. Amolsch's (1937) case is often quoted wrongly in this connection, as this was not a botryoid sarcoma of the vulva, but a metastasizing myxoid sarcoma, possibly a fibrosarcoma, of the vulval subcutis.

Considering the identical structure of botryoid sarcomas arising in such different sites as the nasopharynx and other areas of the head, and in the common bile duct, as well as in the pelvic organs, it is difficult to understand why many authors have, and are still adhering to the tenet that botryoid sarcomas of the urogenital tract can only be derived from elements of the Müllerian or Wolffian ducts. Willis (1948) has discussed the unlikelihood of such theories.

The histological picture of botryoid sarcoma is uniform and monotonous. It presents as a small-celled undifferentiated mesenchymal neoplasm, the cells of which, at least in the primary growth, are usually separated by much oedematous fluid. According to some observers mucins may be demonstrated in the latter, but in the present case all special mucin stains have been uniformly negative. Below the epithelium of the polypoidal masses the growth tends to be more cellular, forming what has been termed a cambium layer, as it is reminiscent of the cellular zone interposed between the bark and the wood of a tree. Multinucleated tumour giant cells have been occasionally noted in this tumour in adults, but were present in our case and signify probably no more than rapid cell division.

Botryoid sarcomas in children, as already stated, are completely anaplastic tumours except for the occasional differentiation into rhabdomyoblasts which, incidentally, are rarely present in the advanced stages of the disease. This finding raises the question whether the neoplasm should be regarded as a rhabdomyosarcoma, and this term has in fact been used by some authors. However, reasons can be adduced which make it unlikely that botryoid sarcomas represent striped muscle tumours. In the first place most of them occur in organs and tissues where normally no striated muscle is present. Secondly, the cervical and uterine counterpart in adults may also show differentiation into smooth muscle, fat, cartilage

and bone. This suggests a retained pluripotential property of the growing mesenchyme, which for unknown reasons is limited in the tumours of children to primitive rhabdomyoblasts.

Aberrant differentiation is well known in both epithelial and mesenchymal growths: squamous metaplasia can be observed in cancers of the gut, breast, stomach, uterus, ovary, gallbladder and other organs, adenocarcinomata may arise in bladder and renal pelvis (Willis, 1958), uterine fibroids may be transformed into "fatty fibroids", consisting of adipose tissue only, and much fatty tissue can arise in the stroma of adenomas of the thyroid (Willis, 1948), of the parathyroid (Ober and Kaiser, 1958), and thymic overgrowths (Shillitoe and Goodyear, 1960). Bone and cartilage may be formed in lipomata and other benign growths (Plaut, Salm and Truscott, 1959), in tumours of the urinary tract (Pang, 1958) and of the soft tissues (Salm, 1959). All these different lesions are due to an identical process: benign and malignant epithelium and mesenchyme may, especially when proliferating, lose its original characteristics, become undifferentiated, and, when subsequently differentiating anew, do so in an unusual and unexpected direction. Thus the finding of rhabdomyoblasts in juvenile botryoid sarcoma, and of other mesenchymal constituents in the adult form, can be readily explained along these lines and there would appear to be no need to invoke theories like those of embryonal rests or displaced tissue of embryonal ducts.

The therapy of botryoid sarcoma has remained disappointing. Almost all workers agree that the neoplasm is radioresistant and thus radium and radiotherapy are of no avail. Early, adequate, and sometimes heroic surgery appears to be the only way to preserve life. Thus Ober, Smith and Rouillard (1958) reported 2 cases of congenital vaginal tumours. In both girls, at the age of about 2 weeks, the uterus was excised together with the entire vagina, and both patients were alive and well 2 years after the operation. Daniel *et al.* (1959) reviewed a series of 13 cases with 2 successes. One, a girl aged 10 months at the time of the operation, was alive and well 2 years later; the other, a woman of 21 years, had remained well for 5 years. In both patients a total pelvic exenteration had been performed, with bilateral uretero-sigmoidostomy. But it is evident that the prognosis in most cases is unfavourable.

SUMMARY

A case of vaginal botryoid sarcoma in a girl of 2 years, who died 7 months after the onset of the disease, is presented. A complete necropsy showed much direct and coelomic spread, with massive metastatic tumour formation in omentum and pouch of Douglas, as well as considerable lymphatic and haematogenous dissemination.

In addition to a macroscopical tendency to polypoidal growth there was evidence of polypoidal ingrowths into microscopical preformed cavities, such as lymphatic sinusoids, bronchi and lung alveoli.

The relevant literature is reviewed and the similarity is stressed between botryoid sarcomas of the urogenital region and certain tumours of other sites, especially nasopharynx, middle and external ear and other areas of the head, and of the extrahepatic biliary ducts.

As botryoid sarcomas in the pelvic region arise in the organs of the lower urogenital system, hymen and anus, it must be held that the tumour is formed by

tissues derived from the embryonal cloaca, and the occurrence of rhabdomyoblasts and other mesenchymal elements is interpreted as aberrant differentiation.

The therapy is briefly discussed.

I am most grateful to Professor R. A. Willis for reading the manuscript and for his constructive criticisms. I am indebted to Miss Phyllis E. Coleman for the photographic work and to Mr. J. G. H. Ince for the clinical data and for arranging the autopsy.

REFERENCES

Amolsch, A. L.—(1937) *Arch. Path.*, **24**, 777.
Cappell, D. F. and Montgomery, G. L.—(1937) *J. Path. Bact.*, **44**, 517.
Daniel, W. W., Koss, L. G. and Brunschwig, A.—(1959) *Cancer*, **12**, 74.
Duncan, A. S., and Fahmy, E. C.—(1953) *J. Obstet. Gynaec., Brit. Emp.*, **60**, 87.
Edwards, A. C., Sheboygan, W. and Richardson, A. L.—(1933) *Amer. J. Obstet. Gynec.*, **27**, 896.
Eibergen, R.—(1952) *Ned. Tijdschr. Geneesk.*, **96**, 1761.
Farinacci, C. J., Fairchild, J. P., Sulak, M. H. and Gilpatrick, C. W.—(1956) *Cancer*, **9**, 408.
Hanbury, W. J.—(1952) *J. Path. Bact.*, **64**, 763.
Horn, R. C. and Enterline, H. T.—(1958) *Cancer*, **11**, 181.
Idem, Yakovac, W. C., Kaye, R. and Koop, C. E.—(1955) *Ibid.*, **8**, 468.
Macgregor, A. R.—(1960) 'Pathology of Infancy and Childhood'. Edinburgh and London (Livingstone), p. 505.
Maconie, A. C.—(1944) *J. Laryng.*, **59**, 32.
Martin, G. E. and Alexander, W. A.—(1924) *Ibid.*, **34**, 312.
Mostofi, F. K. and Morse, W. H.—(1952) *J. Urol.*, **67**, 681.
Nicory, C.—(1923) *Brit. J. Surg.*, **11**, 218.
Ober, W. B. and Edgcomb, J. H.—(1954) *Cancer*, **7**, 75.
Idem and Kaiser, G. A.—(1958) *Ibid.*, **11**, 601.
Idem, Smith, J. A. and Rouillard, F. C.—(1958) *Ibid.*, **11**, 620.
Pang, L. S. C.—(1958) *J. Path. Bact.*, **76**, 357.
Plaut, G. S., Salm, R. and Truscott, D. E.—(1959) *Ibid.*, **78**, 292.
Prior, J. T. and Stoner, L. R.—(1957) *Cancer*, **10**, 957.
St. John, E. G. and Wood, Z. P.—(1955) *Radiology*, **65**, 218.
Salm, R.—(1959) *Brit. J. Cancer*, **13**, 614.
Sharp, W. C. and Helwig, E. B.—(1959) *Amer. J. Dis. Child.*, **97**, 845.
Shillitoe, A. J. and Goodyear, J. E.—(1960) *J. Clin. Path.*, **13**, 297.
Söderberg, F.—(1933) *Acta otolaryng., Stockh.*, **18**, 453.
Stobbe, G. D. and Dargeon, H. W.—(1950) *Cancer*, **3**, 826.
Taylor, C. W.—(1958) *J. Obstet. Gynaec., Brit. Emp.*, **65**, 177.
Ulfelder, H. and Quan, S. H.—(1947) *Surg. Clin. N. Amer.*, **27**, 1240.
Willis, R. A.—(1948) 'Pathology of Tumours'. London (Butterworth), p. 754.—(1958) 'The Borderland of Embryology and Pathology'. London (Butterworth), pp. 516 and 555.

CANCER OF THE OESOPHAGUS IN SINGAPORE

YAHYA COHEN AND SYLVIA HOE

From the Department of Surgery, University of Malaya

Received for publication February 27, 1961

SCOPE in the treatment of cancer of the oesophagus has increased enormously in the last few years. This has been due not only to improved techniques of attack upon this organ—techniques which are now standardized and universally accepted —but to the great advances of the last decade in anaesthesia, antibiotic therapy, nutritional states and fluid balance. As in all branches of surgery, availability of treatment in a particular condition stimulates the study of it. This is perhaps more true of carcinoma of the oesophagus than most of the surgical conditions in Singapore since it offers problems which were seemingly insurmountable not many years ago.

The object of this study is to review all the cases of cancer of the oesophagus admitted to the Surgical Professorial Unit of the Civil General Hospital, Singapore, between the years 1948–57 and to study the pattern of this disease as it occurs here. In all, one hundred and seventy cases were admitted into this unit in this decade. There has been a paucity of records in the immediate post-war years but this has not detracted from the reliability of such analysis as has been done.

Malignant lesions of the oesophagus occur throughout its whole length but when originating primarily in this organ take the form of a squamous cell carcinoma. Adenocarcinomas arising *ab initio* from the oesophagus are unusual if not rare. However, many cases of adenocarcinoma in the lower third are often encountered. These are almost invariably growths of the stomach involving the lower end of the oesophagus. Such growths may spread to involve a considerable section of the distal oesophagus. Adenocarcinoma of the lower third can arise from heterotopic gastric tissue found there but it is difficult to establish that such lesions have in fact arisen in the oesophagus. The fundus of the stomach shares in this involvement and it is more than likely that such growths originate there. For purposes of this study only carcinoma of the oesophagus has been analysed.

Hospital incidence

Analysis of these one hundred and seventy cases admitted in the last ten years shows a steady increase in numbers as is shown in Fig. 1. There is no specific reason for this increase which appears to keep pace with a similar increase in the number of patients seeking hospital treatment in this period, as shown in Fig. 2.

An analysis of cases of cancer of various organs was made from figures obtained from the Annual Hospital Reports between the years 1954 and 1957. Figures before this period were not taken into consideration because cancers of the oesophagus were then included with those of cancers of the oral cavity and pharynx.

It is interesting to note that this is the fifth most common malignant lesion found in Singapore hospitals. These figures are significant in that they include all

Colony of Singapore Annual Reports
1954–1957

Organ	Total numbers	Percentage
1. Cervix uteri	1014	20.05
2. Stomach	653	12·91
3. Trachea, bronchus and lung	567	11·21
4. Buccal cavity and pharynx	447	8·84
5. Oesophagus	390	7·71
6. Liver	371	7·34
7. Breast	240	4·77
8. Rectum	189	2·73
9. Large intestine	137	2·70

cases admitted to all hospitals and units in Singapore. Carcinoma of the oesophagus has a higher incidence than carcinoma of the liver or breast. It is likely, however, that the figure of 7·71 per cent includes a large number of gastric carcinomas which involve the oesophagus. It is reasonable to assume that most of the diagnoses were made on clinical or radiological evidence and this form of assessment is quite unreliable in determining the origin of the growth for reasons already mentioned.

Racial incidence

It has always been an impression that in Singapore this lesion is more common among the Chinese. This impression is borne out by our studies.

Table I shows that no less than 95·3 per cent of our patients were Chinese. The percentage of Chinese in hospital admissions covering this ten-year period is 74·17 per cent. This tallies with the percentage of this community in the general population (75 per cent). The excessive incidence in this race therefore cannot be explained by their greater desire for hospital treatment.

TABLE I.—*Racial Incidence and Hospital Admissions*

Race	Cases of cancer of the oesophagus		All hospital admissions, 1948–57	
	Number	Percentage	Number	Percentage
Chinese	162	95·3	414,533	74·17
Indians	7	4·1	83,078	14·86
Malays	1	0·6	32,085	5·74
Europeans	0	0	12,755	2·28

This apparently greater incidence however requires further clarification and may be due to other factors.

Table II shows the rate of incidence per ten thousand admissions in two major age groups in either sex.

TABLE II.—*Rates Per* 10,000 *Admissions*

Age group	Chinese		Indians	
	M.	F.	M.	F.
25–54	22·48	5·38	3·82	9·80*
55–80	54·22	38·71	0·08*	0

* Figure based on only one case.

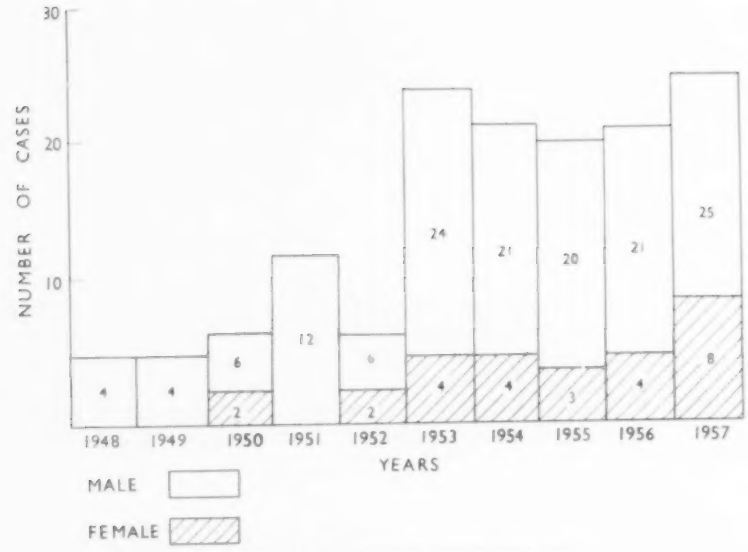


FIG. 1.—Hospital incidence of oesophageal cancer.

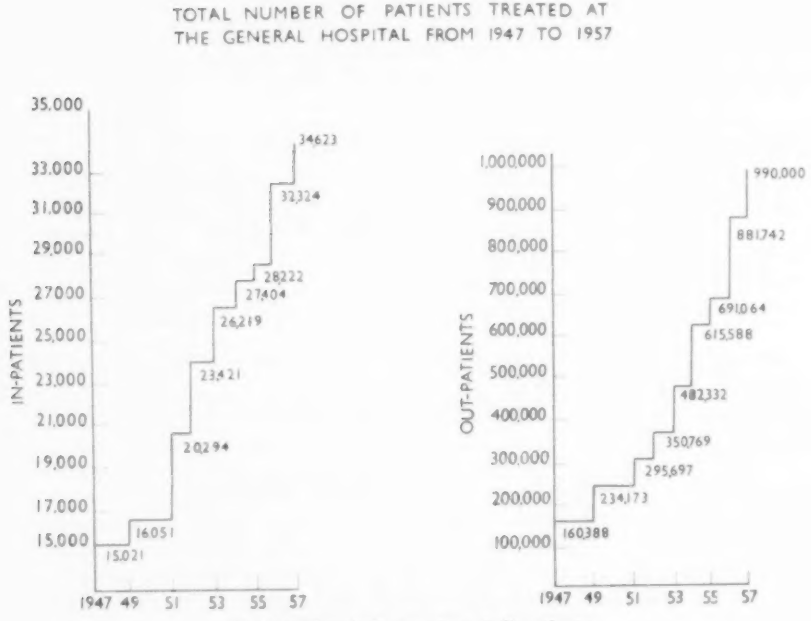


FIG. 2.—Hospital reception of all patients.

This breakdown shows that the incidence of cancer of the oesophagus amongst Chinese males between the ages of twenty-five and fifty-four is some seven times that of Indians in the same age group.

In the older age group between fifty-five and eighty the incidence is some five times. This however cannot survive critical scrutiny as there was only one case in the Indian group. This also applies to the figures for females which are too small to be subjected to statistical analysis.

There is an interesting reflection in the breakdown of hospital admissions by age, sex and race between the years 1955–57. This shows that in the first age group (25–54 years) there were 48·8 per cent Chinese males and 77·0 per cent Indian males; whereas in the older age group (55–80 years) there were 17·9 per cent Chinese as opposed to 9·4 per cent Indian males.

We are unable to find a cause for the susceptibility of the Chinese to this disease. It has been suggested that swallowing very hot food could be a possible source of irritation to the oesophagus. There is insufficient data to show whether this impression is correct. Nor can any relationship be found with personal habits such as smoking and drinking.

Sex incidence

Of the total number of one hundred and seventy cases analysed there were one hundred and fifty-three males and twenty-seven females. This gives an overall ratio of five to one. These figures substantiate the statement made by British workers that cancer of the oesophagus is a disease of males. Aird (1957) gives an overall ratio of five males to one female and Franklin (1952) a ratio of four to one. Study of Table II, however, shows that in the Chinese subjects the rate of incidence in males to females in the first age group (25–54 years) is four to one whereas in the older age group the ratio is about 1·5 to 1.

Figures for Indian subjects were too small to be subjected to analysis.

Age incidence

Fig. 3 indicates the age distribution of the disease as found in this series.

It is not possible to deduce the relative incidence of the disease in the various age groups as no figures for hospital admissions in these groups are available for these ten years. Nor can these figures be compared with those given for the United Kingdom as the expectation of life in Singapore is lower.

The ages range from twenty-five years to seventy-four years. The average age was 49·36 years.

The youngest subjects were a Malay female and an Indian male both aged twenty-five years with growths involving the lower third of the oesophagus. The next youngest was an Indian male aged thirty with a growth of the hypopharynx which had extended into the superior mediastinum.

The oldest subjects were aged seventy-one, seventy-two and seventy-four years all with growths involving the lower third.

Social status and occupation

Parker, Hanna and Postlethwaite (1952) in a study of one hundred and seventy American cases in South Carolina show an overwhelming preponderance of this disease amongst members of the lower economic groups. In their practice, two

out of every three patients were private cases as opposed to "service or clinic patients". On the other hand only 4 per cent of the cases of carcinoma of oesophagus were private patients.

During the period of our study, fifteen of the one hundred and seventy cases were "private" patients. This constitutes 8·82 per cent of the total number. In the year 1957, for example, nine hundred and eleven of the five thousand five hundred and seventy-two admissions went into the "private" wards. This constitutes 16·4 per cent. We consider this significant in supporting the suggestion

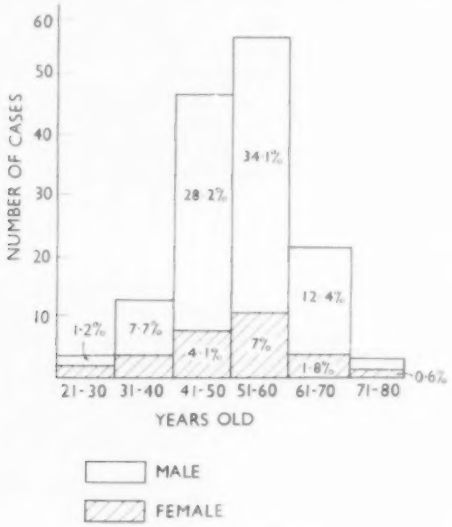


FIG. 3.—Age incidence.

of these workers that this disease occurs more commonly among people of the lower income groups. No reason can yet be suggested for this.

We did not find it possible to establish a relationship between occupations and this disease. Most of the members of the lower income group were classified as housewives or labourers, facts that did not allow of more definitive analysis.

Symptomatology

Dysphagia

By far the commonest complaint was dysphagia—this symptom being recorded in one hundred and sixty two cases (95·29 per cent). The remaining eight, complained primarily of pain, vomiting, hoarseness, loss of weight, cough or haemoptysis. One patient presented with a secondary in the right mandible.

The overwhelming preponderance of this symptom makes it urgent that any case of dysphagia must be investigated for carcinoma of the oesophagus. This fact has, of course, been stated by many authors but is always worth reiterating in view of its immense significance in this disease. Barium meal studies must not be considered to be adequate as false negative findings are not uncommon especially

in the lower end. False positives may appear especially in the upper end. Oesophagoscopy is essential and should be repeated if findings are negative or inconclusive or if the symptoms persist.

The dysphagia was by no means always progressive and was intermittent in no less than 12·27 per cent of the cases. We consider this figure significant and the proportion high. The spontaneous relief from this symptom may therefore only be evidence of this intermittency and should not be considered to be unimportant. It is clear that the symptom would be more persistent and progressive when the growth is large and protrudes into the lumen of the oesophagus. The more infiltrative and therefore more dangerous type of growth would make itself less apparent through the progressiveness or intensity of this particular symptom.

Intermittency may be ascribed to a number of causes. There may, for example, be a sloughing of the growth—a phenomenon that is sometimes witnessed on oesophagoscopy. The oesophagus may accommodate itself to the conditions imposed by the growth and this may give rise to alleviation of symptoms from time to time. It is possible too, that the patient may become adapted to a certain degree of dysphagia and only experience this symptom again when constriction becomes more severe.

Of the one hundred and sixty-two cases who complained of dysphagia, fifty-six or 34·5 per cent said that the dysphagia was complete by the time they were admitted. Complete dysphagia connotes inability to swallow all manner of food whether solid, semi-solid or liquid. In one hundred and three cases or 63·5 per cent this dysphagia was incomplete, the patients being able to swallow semi-solids such as rice porridge, bread or biscuits dunked in milk or liquids. Sixty-seven of these cases were able to swallow only liquids. In three cases the degree of dysphagia was not recorded.

Duration of the dysphagia varied from twenty days to one year. In one case the symptoms had lasted four years but this was a case of a Plummer-Vinson syndrome with malignant change. The dysphagia must be attributed to the syndrome. We were able to confirm the work of others that duration of dysphagia was not related to resectability as is shown in Fig. 4. Nor indeed does it affect operability and survival after operation. One case where the dysphagia had lasted over a year was found to be eminently operable and the patient is still alive four and a half years after operation.

The average period of dysphagia was 4·1 months.

It has often been stated that the site of discomfort usually corresponds with the site of the growth. We are unable to confirm this. Only one hundred and three cases could be analysed from this point of view but this was enough to show that there is considerable variability in this relationship. Table III shows this clearly and it is of interest to note that in a proportion of cases the site described as indicated by the patient was distal to the site of the lesion.

No reliance can be placed therefore on the evidence of the patient as to the actual site of the lesion.

Loss of weight

This was the next most common symptom and was noted in one hundred and forty-one of our cases (82·94 per cent). In twenty-five cases (14·7 per cent) this was not noted and in four there had been no loss of weight at all. Of those who complained of weight loss eighty per cent stated that this was considerable.

TABLE III.—*Site of Discomfort in Relation to Site of Lesion in* 103 *Cases*

Site of lesion	Number of cases	Site of discomfort				
		Neck	Upper sternum	Mid sternum	Lower sternum	Epi-gastrium
Upper third	13	8	—	3	2	—
Middle third	65	18	13	14	12	8
Lower third	25	5	3	3	12	2

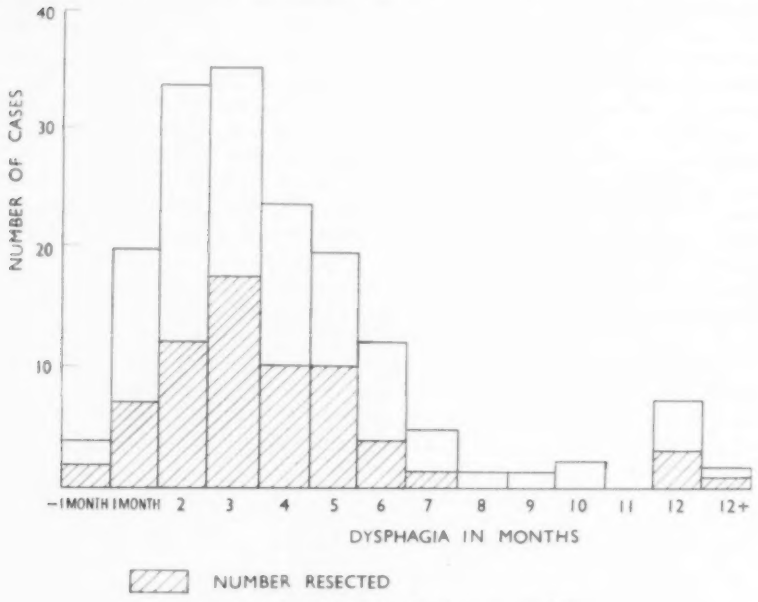


FIG. 4.—Relationship of dysphagia to resectability.

Vomiting and regurgitation

One hundred and thirty patients (76·47 per cent) complained of vomiting. Fourteen patients stated that they definitely did not suffer from this symptom. In the remainder this symptom was not recorded. In many cases there was no true vomiting but a regurgitation of oesophageal contents. The process varied considerably. In some there was a return of food mixed with saliva and mucus. With others there was an efflux only of saliva and mucus which occured after the intake of food. This was immediate in some cases and was delayed up to about two hours in others.

Other symptoms

The three cardinal symptoms of dysphagia, loss of weight and "vomiting" by far exceeded all other symptoms which were :

Salivation	Hoarseness	Pain	Haematemesis
Cough	Weakness	Swellings in neck	Dyspnoea
	Constipation		Stridor

Cough may be a symptom of some significance. Three cases with this complaint had an oesophago-bronchial fistula and with these, cough was a leading complaint and occurred on swallowing liquids. Other causes of cough were lung abscess, mediastinal secondaries or active pulmonary tuberculosis. In some the cough was associated with emphysema.

Hoarseness was present in thirteen cases and was due to recurrent nerve paralysis. This was always assumed to be a sign of advanced disease. Some of these subjects had involvement of the supraclavicular and mediastinal lymph nodes.

Weakness was not a common symptom, occurring in only thirty-seven cases. Apart from those who were admitted *in extremis* weakness as a symptom was seldom offered as a primary complaint. This is suprising as it is well known that "weakness" is a common complaint among Chinese patients. It is probable therefore that the other symptoms were more pressing in this condition or that these patients accepted weakness as a natural consequence of their inability to eat and did not think it worth mentioning.

It was not surprising that fifty-eight or 34 per cent of these patients complained of *constipation*. Certainly the dehydration from which so many suffered was a contributory factor as well as the lack of bulk and a sluggishness of the gastro-colic reflexes.

Although *enlargement of lymph nodes* was found in 13 per cent of cases, only one or two patients complained of it and this will therefore be discussed with the physical findings.

Pain as such was hardly ever offered as a symptom and was conspicuous by its rarity.

Seven patients complained of *dypsnoea*, and in one this could be attributed to mitral disease. The others had mediastinal involvement of lymph nodes or oesophago-bronchial fistula.

One patient who complained of *stridor* had massive involvement of the mediastinal and cervical lymph nodes.

Two cases had *haematemesis* before admission. In both there was a history of epigastric pains typical of peptic ulceration.

Salivation was only offered in two cases. In these two the excessive salivation was uncomfortable and required much spitting. In one case the growth was in the upper third and in the other in the middle third of the oesophagus.

Physical Signs

This disease is characterized by a paucity of physical signs. Little help is to be expected from these in assessing operability or even resectability. Except for obvious manifestations such as involvement of lymph nodes, liver, presence of ascites, etc., such assessment can only be made at operation.

Patients' general condition

Observations on the patients' general condition were made in all but twelve or 7·05 per cent of cases. These were made in terms of whether the patient's general condition was good, fair or poor. It is realized that this is only a personal impression of the individual clerking the case but is information which is of great sig-

nificance. Of the one hundred and fifty-eight patients on whom this observation was made the analysis was as follows :

	Number	Percentage
Good	28	17·72
Fair	72	45·5
Poor	58	36·71

It would appear that a considerable proportion of patients were in fair condition when admitted. However, forty-seven patients were described as being emaciated.

A large number of the patients were barrel-chested and emphysematous. This may be attributed to the age group in which most of these lesions occur.

Dehydration

This, as adjudged clinically by loss of elasticity of skin, sunken eyeballs and a dry tongue, was found in about half the cases. In about one-third of the cases the dehydration was described as severe.

Fever

Elevation of temperature was noted in twenty-seven or 15·97 per cent of the cases. In most this was a slight elevation and was intermittent in nature. In three cases this temperature was high and suggested a septic process. One had a lung abscess due to an oesophago-bronchial fistula. The second had a fistula with pneumonia. The third and most interesting of the three had a dual growth in the mid-oesophagus and stomach. Biopsies of both showed squamous cell carcinoma. His temperature remained swinging in nature until his death three months later. It is presumed that the spread was by implantation from the primary in the middle third of the oesophagus. Unfortunately, permission for autopsy could not be obtained. We feel that the temperature here was due to the ulceration and infection of the extensive growth in his fundus. Similar swinging temperatures have been seen in fungating massive gastric carcinomas. These temperatures had settled after resection of such growths.

It has often been stated that the presence of fever in thoracic oesophageal neoplasms suggests advanced disease, ulceration and infection. Apart from cases where there is a swinging temperature and obvious cause for this we do not consider the presence of a low grade temperature as a contraindication to exploration and the performance of palliative procedures.

Involvement of cervical lymph nodes

Nine cases showed definite involvement of cervical nodes. Criteria of involvement were enlargement, hardness and fixity. Cases where lymph nodes were merely palpable, soft or discrete are not included. Biopsy was done only in one case and showed secondary involvement.

The distribution of cervical lymph node involvement was shown as follows :

	Cases	Involvement	Percentage
Upper third	14	1	7·1
Middle third	103	6	5·8
Lower third	47	2	4·3

Concomitant Disease

In only nine cases was there any serious concomitant disease. Six of these cases had active pulmonary tuberculosis and in five the disease was bilateral.

One patient had a highly positive Kahn test.

One patient was an established case of mitral stenosis with early heart failure.

There was one case with an enlarged prostate who had an acute urinary retention following gastrostomy.

Special Examinations

Barium swallow

This is a routine examination in all cases of dysphagia and invariably the first of all special examinations. Attention has already been drawn to the occasional unreliability of this examination which should always be followed by endoscopic verification and biopsy. Artefacts are occasionally produced by air bubbles, spasm of the oesophagus or the presence of food particles so as to give a completely wrong impression of the character and extent of the lesion. Further, a normal barium swallow in the presence of dysphagia does not always exclude a carcinoma. In either case a repeat barium swallow is always worth doing especially if there is difficulty in associating symptoms with the X-ray studies or *vice versa*.

Occasionally benign lesions of the oesophagus such as achalasia, tuberculosis, reflux oesophagitis or varices, produce impressions not unlike those of a carcinoma. A barium swallow in the Trendelenburg position using a thin barium mixture has value in assessing the lower radiological limit of the lesion accurately. The Trendelenburg position is useful in outlining small lesions of the oesophagus not detectable in the erect position due to the rapid passage of the barium.

Nearly all cases under review had a barium swallow. The exceptions were those few cases who were admitted *in extremis* and were unfit for this. Six such cases were encountered in this series.

Endoscopic examinations

Oesophagoscopy did not become a standard method of examination in Singapore until recently. The reason for this was the fact that resectional surgery was not often attempted in the earlier period.

In all, sixty-seven cases in the series underwent this examination. It is our opinion that oesophagoscopy should always be done under a general rather than a local anaesthetic. It is pleasanter for the patient and gives the endoscopist a full opportunity to carry out a thorough examination in a relaxed patient, especially as in some growths of the lower third considerable time may be spent in clearing food debris. The occasional nervous and excitable patient may, by his restlessness, make this investigation fraught with danger.

Bronchoscopy can be of value in determining involvement of the bronchus Oedema of the bronchial mucosa may be suggestive of growth in close proximity to the bronchial wall. Distortion of the trachea, carina or bronchi may suggest mediastinal lymph node involvement.

Pathology

There is unfortunately insufficient data on the pathological findings to give us reliable study data on the macroscopic and microscopic characteristics of our cases.

Some of the figures available to us, however, are worthy of study if only for the formulation of a general impression of the disease as it exists in Singapore and as a reference to future and more accurate studies that may be made in this field.

Site

It has been traditional to classify growths of the oesophagus as occurring in the upper, middle and lower thirds. The upper third of this organ extends from the cricoid cartilage to the arch of the aorta or vena azygos lying at the level of the fourth thoracic vertebra. This is twenty-five centimetres, from the incisor teeth. The middle third extends to the level of the inferior pulmonary vein. The rest of the oesophagus extends to the cardio-oesophageal junction and is described as the lower third.

This classification gives only a general and, at best, rough impression as to the whereabouts of the neoplasm. Hardly any indication is given of the extent of the growth which may be a point of supreme importance in the operative management of the disease. A small percentage of the tumours (5·7 per cent) occur above the arch of the aorta but are confined to the thorax and are sometimes limited in their upper extent to the level of the dome of the cervical pleura. It could be misleading to describe these tumours as belonging to the upper third as this often suggests a post-cricoid or cervical growth. Some tumours extend from the bifurcation of the trachea to the cardia and cannot correctly be described as being either middle or lower third growths. Limited tumours occurring at the junction of these sites also cause similar difficulties in description.

Figures from various sources show considerable variation on the site of these tumours and there can be little doubt that this variation at any rate, may be partly attributed to the inaccuracy of such classification.

	Kaufman (1929) (%)	British Empire Cancer Campaign (1942) (%)	Franklin (1952) (%)
Upper third	17·7	12	25
Middle third	34·6	47	45
Lower third	47·7	41	30

For purposes of this study, we classified all tumours lying within the levels described above as belonging to that particular third. Tumours which are supra-aortic therefore fall within the classification of " upper third ". Those, however, that are crossed by the vena azygos through the middle of the growth are included amongst the middle third growths. Where a greater extent of the tumour lies above the vena azygos it was described as belonging to the upper third group. Where a greater extent of the tumour lies below the vena azygos it was included in the middle third group.

Neoplasms which cause special difficulty in classification are those which infiltrate the middle and lower thirds of the oesophagus. We have preferred to

place these in a special group which, for convenience, we have called the lower half. This special category is necessary not only because it indicates the great extent of the growth, but because it gives rise to special difficulties in surgical technique.

We have records of the site of this disease in one hundred and sixty-four or 96 per cent of the cases. Their distribution is as follows:

Site	Number of cases	Percentage
Upper third	14	8·2
Middle third	103	60·6
Lower third	39	21·0
Lower half	8	5·0

Siting was based not only on radiological findings but in most cases was confirmed or modified by endoscopy, at operation or autopsy.

Histology

There was only one case showing special interest in the histological findings. This was an adenocarcinoma of the middle third of the oesophagus. This tumour was confined to this segment and did not involve the lower third.

Treatment

Radical cancer surgery of the oesophagus has only been attempted in Singapore as a routine procedure in recent years. Difficulties that assailed surgeons in the past have already been mentioned. In the earlier period the shortage of ancillary surgical aids such as anaesthesia, blood transfusions, physiotherapy, was too great and discouraged attempts at radical excision.

Patients came into hospital when they were too ill to withstand any but the minimum of palliative procedures. There has been in recent years a growing medical consciousness in the average patient in Singapore which has resulted in his seeking medical aid in an earlier period of his illness. The confidence in modern methods of surgical treatment is reflected not only in this lesion but in all diseases. This has resulted in a steady growth in the scope of surgical effort and a similar steady improvement in results.

It would not be altogether irrelevant here to give credit to those early workers who attempted such surgery as was done in spite of the difficult conditions that existed. It is to their efforts made against great odds and to their tenacity of purpose that we owe such as can be accomplished today.

A review therefore of the way in which all these cases were managed is not only of value as a base line for future studies, but gives, in some measure, a historical background to a changing surgical scene and advancement in the pattern of management in a form of malignant disease in this country.

Table IV is an analysis of how these cases were treated and the mortality from these procedures.

Perhaps the most significant figure in the above analysis is the high mortality in cases where no treatment for the relief of dysphagia was undertaken. It is a reflection of the desperate states in which some of these patients came into hospital. Of the twelve cases which come into this category, six died within a few days after admission. The seventh death was a most unusual case of a metastasis in the mandible. The primary was unknown at the time. This patient survived

TABLE IV.—*Total Number*—170

Type of treatment	Number of cases	Percentage of cases	Mortality	
			Number	Percentage
Absconded	19	11·2	—	—
No treatment	12	7·1	7	58·3
Souttar's Tube	8	4·7	1	12·5
Radiotherapy	1	0·5	—	—
Gastrostomy	31	18·2	4	12·9
Jejunostomy	11	6·5	8	72·7
Explored only	19	11·2	1	5·2
Resection	69	40·6	31	44·9

hemi-mandibulectomy but died on the fourteenth day of cerebral metastases. A primary squamous cell carcinoma of the middle third of the oesophagus was discovered at autopsy.

Of the remaining five cases on whom no treatment was undertaken, two were cancers of the upper third considered inoperable. The third had massive secondaries in the mediastinum causing pressure symptoms. The fourth had a tracheostomy because of respiratory obstruction and the fifth had a strongly positive Kahn test.

Significant too, is the fact that a simple palliative measure such as jejunostomy also carried a high mortality. It was presumably not possible to carry out a gastrostomy on these cases. It is clear therefore that jejunostomy serves no useful purpose as a supportive measure.

The rate of resectional surgery has risen steadily since 1952 and cases where only an exploration was done have decreased accordingly as shown in Fig. 5.

This is due to an increasing appreciation of palliative resection where growth is left behind and where a varying mass of tumour tissue is removed and continuity of the upper alimentary tract restored. This brings about a more comfortable end than that experienced from a slow and protracted starvation.

It will be seen from Fig. 5 that mortalities fell steadily between the years 1953 and 1955 as experience was gained in this field of work. There was a sharp rise, however, in the following year. This was due to a trial made at modification of the standard procedure for middle third growths. This modification consisted of a synchronous-combined abdomino-thoracic procedure which was attempted during that year. The mortality rate from this procedure was high and did not merit its continuance.

Radiotherapy has hardly been used as an elective method of treatment in Singapore. Studies from special radiotherapy centres suggest that results by these methods have been comparable with those of surgery. Radiotherapy has been available in Singapore only recently and for varying periods. Latterly it has been used as an adjunct to surgery and residual tumours have been irradiated. Recurrences in lymph nodes have responded remarkably to this form of treatment. It is too early however to assess the combined method of treatment from the point of view of an increased length of survival.

Souttar's tubes have only been used since 1954 and have steadily replaced gastrostomy as a method of palliation for inoperable cases. This method has only been used for growths in the upper and middle thirds and is a satisfactory form of palliation because it permits of feeding through normal channels. The insertion of Souttar's tubes was undertaken by the ear, nose and throat surgeons.

Operative methods selected depended on the site of the tumour.

Middle third growths were attacked by the method described by Lewis (1946) where the stomach and lower oesophagus are mobilised by the abdominal route. Resection of the oesophagus is done through a right thoracotomy, the stomach delivered into the pleural cavity through the oesophageal hiatus and continuity restored with an oesophago-gastrostomy.

Lower third growths have been resected through a left thoraco-abdominal incision after the method described by Allison (1942). In mobile growths resection

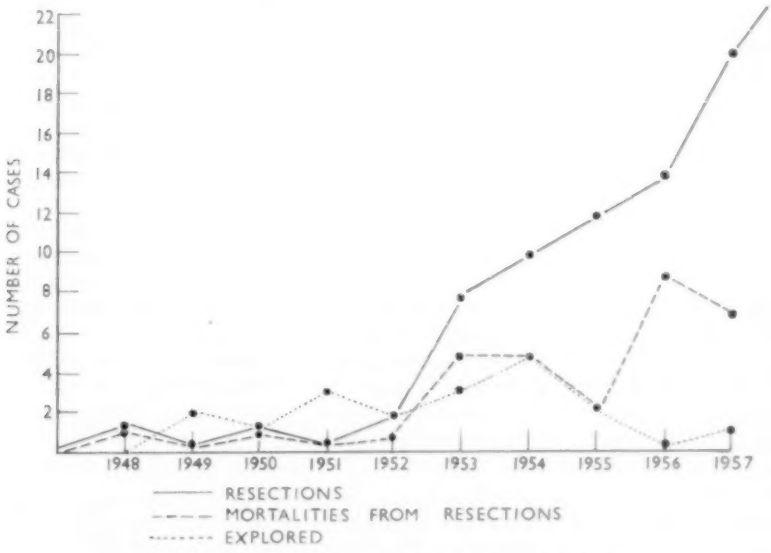


FIG. 5.—Exploration, resection and mortality rates.

is conducted after the principle of a block dissection, removing stomach, oesophagus related parts of greater and lesser omentum, spleen and left half of pancreas and all intervening tissues and lymph nodes in one piece. Continuity is restored by oesophago-gastrostomy or oesophago-jejunostomy.

Supra-aortic growths have been resected by a recently described synchronous-combined abdomino-thoraco-cervical operation done by two surgeons. The stomach is mobilized through a laparotomy. The oesophagus is mobilized through a right thoracotomy, and simultaneously delivered into the neck through a right supra-clavicular transverse incision. Continuity is then effected in the neck by oesophago-oesophagostomy after resection of most of the oesophagus (Yeoh and Cohen, 1958).

It will be noticed from Table IV that of the one hundred and seventy cases sixty-nine were resected. This is 40·6 per cent of all cases. The majority of cases were inoperable and resection was only partial.

Of the sixty-nine cases resected, nine were noted to have uncomplicated, mobile localized tumours without lymph node involvement. These growths were appar-

ently completely excised at operation. Of these one died post-operatively giving a mortality of 11 per cent. We have failed, unfortunately, to trace four cases.

Of the remaining four, two are still alive and well after eighteen months and four and a half years. The other two died ten months and two years after operation. The former developed hoarseness of voice and the latter supraclavicular lymph nodes.

In the remaining sixty cases the growth was either incompletely removed because of fixation to surrounding vital structures or, being mobile and localized, was complicated by secondary involvement of lymph nodes which were incompletely removed. There were thirty post-operative deaths in this group, giving a mortality of 50 per cent. Only sixteen of the thirty survivals could be traced.

These survived from periods ranging from one month after discharge from hospital to fifteen months. One is still alive after twenty-two months. He developed secondaries in the supraclavicular lymph nodes. These were successfully treated with radiotherapy. He remains free from dysphagia and is in fair health.

One case who survived fifteen months developed multiple secondaries in the skin of scalp, abdominal wall and in isolated lymph nodes eight months after operation. He remained free from dysphagia.

We consider that apart from offering a possibility of cure, the mobile uncomplicated growth offers an enormously greater chance of immediate post-operative survival. This is concluded by the great difference in mortality rates in the two groups. It is felt that smooth excision of the mobile tumour as opposed to the difficult dissection of the fixed growth accounts for this.

We feel that resections should be attempted on all cases because it is impossible to tell at operation which cases are likely to survive for any length of time.

Post-operative Complications

Of those who survived operation five or 13 per cent of cases suffered from post-operative complications. Three had wound infections; one developed a right hydrothorax and one a fistula in the anastomosis which responded to continuous suction drainage of the pleural cavity.

Causes of Post-operative Deaths

We were able to obtain autopsies on only thirteen, or 42 per cent of cases. In the remaining cases diagnosis was clinical. The causes of death in those who underwent resection were as follows:

Cause of death	Number of cases
Post-operative shock	2
Pneumonia	5
Pulmonary collapse	5
Pulmonary oedema	3
Gangrene stomach	4
Fistula in anastomosis	6
Empyema	1
Wound infection	2
Cardiac failure	1
Chylothorax	1
Tracheal fistula	1

It is interesting to note that thirteen of the thirty-one deaths were due to lung complications. This is nearly half of all the deaths and emphasizes the need for careful pre- and post-operative physiotherapy. Lung complications are not surprising in view of the fact that these cases are often emphysematous. We are becoming more and more inclined to do tracheostomies for direct suction of secretions at the slightest indication of pulmonary complications.

One case who died of "gangrene of stomach" on the twenty-eighth post-operative day is worthy of special mention. This patient was making smooth post-operative progress and often complained of being hungry. He collapsed and died suddenly at night. Autopsy revealed a right thoracic cavity full of food and there is little doubt that the stomach had disrupted following a large meal which had been smuggled in by the patient's relatives. There was a gangrene of the upper half of the stomach with a clear line of demarcation separating it from a healthy lower half. Doubtless the large meal had caused a severe and sudden distension of the stomach. This in turn had completely obstructed the already limited blood supply to this part of the organ. This indicates that there should be some discretion in eating for a considerable time after operation.

SUMMARY AND CONCLUSIONS

A paper is presented reviewing one hundred and seventy cases of primary cancers of the oesophagus as seen in the Surgical Professorial Unit of the Civil General Hospital, Singapore, between the years 1948 to 1957.

It is shown that this lesion is more common among the Chinese than in the other communities living in Singapore. No reason is ascribed for this difference.

This work confirms that of others that this condition is more common in males.

This lesion is shown to occur more commonly in those of the lower income groups.

A review is made of the symptomatology and the cardinal symptoms of this disease are dysphagia, loss of weight, and regurgitation or vomiting.

It appears that dysphagia occurs as two main types, progressive and intermittent, and reasons are advanced for these two forms.

It is shown that the site of discomfort is not necessarily related to the site of obstruction.

An assessment is made of the general physical condition of these patients and various aspects of their physical states are discussed. It is pointed out that there is a paucity of physical characteristics in this disease.

Methods of special investigation and their value are discussed.

The site and pathology of this tumour are described and mention is made of the weakness of the present mode of classifying these growths as belonging to upper, middle and lower thirds of the oesophagus.

The methods by which these one hundred and seventy cases were managed are discussed. Comment is made on the steadily changing trend towards more radical procedures due to an increase of surgical resources in Singapore. A plea is made for the benefits that accrue from resectional surgery and from the re-establishment of continuity of the alimentary tract. A difference is made between operability and resectability. It is pointed out that resectability in the majority of cases can only be determined at operation and that satisfactory palliation is

obtained even if tumour tissue has to be left behind. It is pointed out that resectability is not dependent on the duration of dysphagia.

The possibility of long term survivals and even cures is considered.

The operative methods used are briefly summarized and mortalities from all the procedures analysed. An enormous difference in immediate post-operative mortality rates is found between those cases where the growth is mobile and easily resected as opposed to those that are fixed and call for considerable dissection. In the former the mortality rate was 11 per cent. In the latter it was 50 per cent.

A review of such cases as have been followed up is made and a résumé is presented of their period of survival and final cause of death.

Post-operative complications and causes of death are reviewed.

We are grateful to Mr. Tye Cho Yook of the Department of Social Medicine and Public Health for his help in the statistical analyses and to Miss Fam Kim Lan for her untiring secretarial help.

REFERENCES

Aird, I.—(1957) 'Companion in Surgical Studies.' Edinburgh. (E. & S. Livingstone.)
Allison, P. R.—(1942) *Brit. J. Surg.*, **30**, 132.
British Empire Cancer Campaign—(1942) *Ann. Rep.*, **19**, 65.
Franklin, R. H.—(1952) 'Surgery of the Oesophagus.' London. (Edward Arnold & Co.)
Kaufman, E.—(1929) 'Pathology for Students and Practitioners.' Philadelphia. (Blackiston), p. 638.
Lewis, I.—(1946) *Brit. J. Surg.*, **34**, 18.
Parker, E. F., Hanna, C. B. and Postlethwaite, R. W.—(1952) *Ann. Surg.*, **135**, 697.
Yeoh, G. S. and Cohen, Y.—(1958) *Aust. N.Z. J. Surg.*, **28**, 18.

DETECTION AND ATTEMPTED ASSAY OF MAMMOTROPHIC ACTIVITY IN WOMEN'S BLOOD AND URINE

L. E. FRASER*, C. C. SPICER†, P. C. WILLIAMS** AND STRETTON YOUNG*

From the Imperial Cancer Research Fund, Divisions of Pathology and of Statistics†, London, W.C.2, and Division of Physiology and Endocrinology**, London, N.W.7*

Received for publication April 8, 1961

In earlier work on the mammotrophic activity present in women's urine, intact weanling male mice of a Swiss Schneider strain (SAS3) were used as test objects (Hadfield and Young, 1956*a*, *b*). The injection of urine or urine extracts into such mice stimulated development of the mammary glands as shown by extension of the duct system and an increase in the number of end buds or clubs. Similar activity was possessed by oestrone and by prolactin.

Gradually the commercially-bred SAS3 mice lost sensitivity under test and they were replaced by mice of the inbred A2G strain. This was the only other strain out of 37 examined in which the mammary glands of weanling males were responsive to both prolactin and urine (Young, 1957).

The mammotrophic activity in urine was believed to be due to protein hormone(s) from the hypophysis and it was therefore considered advisable to continue the work using hypophysectomized mice given partial steroid replacement with oestrone and progesterone (Hadfield, 1957).

The mammary glands of the hypophysectomized immature males of the A2G strain failed to respond to oestrone plus progesterone or to prolactin or urine alone but responded to combined treatment with all three hormones or to urine given with oestrone and progesterone. Serum from normal women was also effective in combination with the two steroid hormones (Hadfield and Young, 1958).

The present paper reports a somewhat unsuccessful attempt to extend this work. We had hoped to be able to measure the mammotrophic activity in human urine and blood, in order to establish normal values and study variations. This has, however, proved virtually impossible with the test system used, because the responsiveness of the mice to prolactin has diminished. Neat urine is now seldom effective in the test and though mammotrophic activity has again been demonstrated unequivocally in women's serum, the amount can be estimated only very roughly.

MATERIALS AND METHODS

Animals

Mice of the A2G strain were bred in our laboratories from random matings of trios replenished every 3–4 generations with fresh stock from the Laboratory Animals Centre.

Males were hypophysectomized when 22–26 days old and 10–15 g. in weight. These were fed on diet 41B supplemented with rolled oats and had access to water throughout. After operation they were kept at a temperature of 78° C. The mice

were weighed at operation and when due for their first injection; any that had gained more than 10 per cent in weight at this time were discarded (cf. Young and Fraser, 1960).

Hormones

Oestrone was dissolved in arachis oil or bought as an aqueous solution (Organon). Progesterone was dissolved in arachis oil or bought as a suspension in an aqueous medium (Laboratories for Applied Biology, Ltd.). Ovine prolactin (Batch No. U10405) and bovine growth hormone (Batch No. R50109), both from the National Institutes of Health, U.S.A., were dissolved in isotonic saline.

Each of the two latter preparations was known to be contaminated by small amounts of other pituitary hormones.

Urine and blood

Samples of urine (Table I) were collected early on the morning when the mice were due for their first injection and were stored at 4° C. during the injection period.

Blood samples (Table II) were left to clot at 37° C. and cooled at 4° C. before the serum was removed. The serum was then stored at 4° C. until needed for test.

Injections

Injections were started 4–7 days after hypophysectomy. The original procedure was for two injections to be given daily for 5 days. Oestrone and progesterone dissolved in arachis oil were injected subcutaneously into one flank each morning and the material to be tested was injected into the other flank. The respective injection sites were reversed for the evening injections. The total doses of hormone were originally: oestrone, 1 μg.; progesterone, 5 mg.; prolactin, 5 i.u. This injection schedule was later simplified and dosages were reduced as explained in the text. The total dose of urine per mouse was 4 ml. and of serum 1 ml. in all tests.

Assessment of mammotrophic activity

The mice were killed and skinned on the morning of the 6th day and the mammary glands fixed and stained *in situ* before being dissected from the pelt and mounted whole. The mammotrophic activity was then measured by counting the number of clubs (swellings of the duct endings more than twice the diameter of the duct itself) and expressing this for each group of animals as the average number of clubs per mouse (Hadfield and Young, 1956*a*).

RESULTS

Tests of normal urine

Samples of urine from 2 normal young women killed all the mice into which they were injected. Three of the remaining 11 samples from 9 young women stimulated significant, though small, increases in club count (Table I). These active samples were not confined to any phase of the menstrual cycle and second samples (MW/2 and JF/2) from 2 of the women producing them were inactive. No activity was demonstrable in 4 samples of urine from post-menopausal women.

TABLE I.—*Testing Urine Samples for Mammotrophic Activity*

Sample	Day of cycle	Club counts ± s.e.$_m$ (deaths/10) Steroids plus urine	Steroids alone	Steroids plus prolactin
Normal Young Non-parous Women				
RC/1	6	11 ± 4 (1)**	3 ± 1 (1)	36 ± 7 (0)
MW/1	20	11 ± 4 (1)**		
LF/1	20	5 ± 2 (1)	6 ± 2 (0)	53 ± 13 (0)
JF/1	19	14 ± 4 (1)**		
MW/2	5	4 ± 2 (4)	5 ± 1 (0)	41 ± 12 (0)
JF/2	26	6 ± 2 (0)		
EF/1	?	1 ± 1 (3)	8 ± 3 (0)	44 ± 7 (0)
AN/1	19	6 ± 6 (5)		
JB/1	10	4 ± 3 (3)	8 ± 3 (0)	44 ± 11 (1)
JM/1	2	4 ± 3 (6)		
KW/1	38	2 ± 1 (0)	5 ± 2 (0)	51 ± 11 (1)
AM/1	20	4 ± 2 (3)		
MR/1	20	4 ± 1 (4)	11 ± 4 (0)	42 ± 10 (0)
Post-menopausal Hospital Patients				
Bri/1	—	5 ± 2 (4)	5 ± 3 (0)	54 ± 13 (2)
Den/1	—	7 ± 4 (0)		
Tho/1	—	2 ± 1 (2)		
Bed/1	—	7 ± 2 (1)		
Total number of mice		131	79	76
Overall mean		6·0 ± 0·9	6·4 ± 1·0	45·3 ± 3·6

** Significantly different ($P < 0·05$) from response to steroids alone.

This partial failure to confirm earlier results (Hadfield, 1957) could be attributed to diminished sensitivity of the mice for whereas the club count per mouse injected with prolactin had been 80–100 previously, it had now fallen to 36–54. Because of this it was decided to see if the responsiveness of the mice could be improved by modifications of the method of assay.

Simplified test procedure

The experiments outlined in the Appendix showed that the mammary response to prolactin was the same as in the original procedure if a smaller total dose of progesterone (1 mg.) was given as a single injection of an aqueous suspension on the first day of the test and if a smaller total dose of oestrone (0·1 μg.) was given twice daily as before but in aqueous solution. The aqueous vehicle greatly eased the dissection of the mammary glands from the pelt.

Using this simplified procedure, the dose : response relation for prolactin was almost exactly what it had been using the original procedure (Fig. 1) though the response to growth hormone was not so satisfactory. Variability of response from mouse to mouse was still, however, as great as ever.

Tests of normal serum

Altogether 13 samples of serum from normal women, 10 of whom were pregnant, were injected in a total dose of 1 ml. per mouse in combination with the simplified steroid dosage. Three of the samples from pregnant women were too

toxic for any estimate of mammotrophic activity to be made: they killed all, or all but one, of the mice they were injected into. The test mice seem to vary in their resilience as well as in their mammary responses to serum injections—one sample (deS) which killed all the mice when first injected killed only one out of six when the rest of the sample was tested a fortnight later. As the repeat test had a positive result (Table II), some mammotrophic activity must be retained in serum during a fortnight of storage at 4° C.

TABLE II.—*Mammotrophic Activity in Women's Serum*

Subject	Week of pregnancy	Club count ± s.e.m (Number of mice dead in test) Steroids alone	Steroids + serum	Steroids + prolactin (1 i.u.)
McG	26	11±5 (1/10)	31±14 (5/10)	61±13 (1/10)
Col	31		45±17** (1/6)	
Mi	38		35±6** (2/10)	
deS	19	5±2 (4/10)	21±9* (1/6)	36±7 (3/10)
So	11		14±3** (1/6)	
Li	38		31±4* (3/8)	
Qu	15		37±13*** (7/10)	
Number of mice		15	36	16
Overall mean		9±3	30±4***	50±8
LF	Not pregnant	10±4 (0/10)	29±9** (9/13)	43±6 (0/10)
FC			25±3** (6/12)	
JC			32±6*** (2/9)	
Number of mice		10	17	..
Overall mean		10±4	29±3***	..

* Significantly different from response to steroids alone ($P < 0{\cdot}1$*, $< 0{\cdot}05$**, $< 0{\cdot}01$***).

Six of the 7 samples from pregnant women that were tested and all 3 of the samples from non-pregnant women significantly increased the club count (Table II).

DISCUSSION

Mammotrophic activity is used here in a limited and purely empirical sense: it refers to the capacity to increase the club count in the mammary glands of hypophysectomized weanling male mice of a particular strain when the mice are treated with oestrone and progesterone in addition to the material under test.

Our results confirm the earlier evidence (Hadfield, 1957; Hadfield and Young, 1958) that such activity is a property of prolactin and of growth hormone and that it is present in the urine and serum from normal women. Not unexpectedly, we find it is also present in the serum from pregnant women.

The activity found in normal serum would represent about 0·5–1 i.u. of prolactin or 0·05–0·1 mg. of growth hormone per ml. Current estimates suggest that normal blood contains 0·002–0·2 i.u. of prolactin (Simkin and Goodart, 1960) and 0·2 μg. of growth hormone (Read and Bryan, 1960) per ml. Thus it is unlikely that either hormone is alone responsible for the mammotrophic activity in serum. It is much more probable that the activity represents the resultant effect of both

hormones and possibly of those other hormones that are concerned in mammary development in rats and mice (Lyons, 1958 ; Nandi, 1959).

The results in Table II show that the method we have used is adequate for demonstrating the presence of mammotrophic activity in serum. It is not so satisfactory when applied to neat urine though when the reduced sensitivity of the mice is taken into consideration we regard the three positive results in Table I

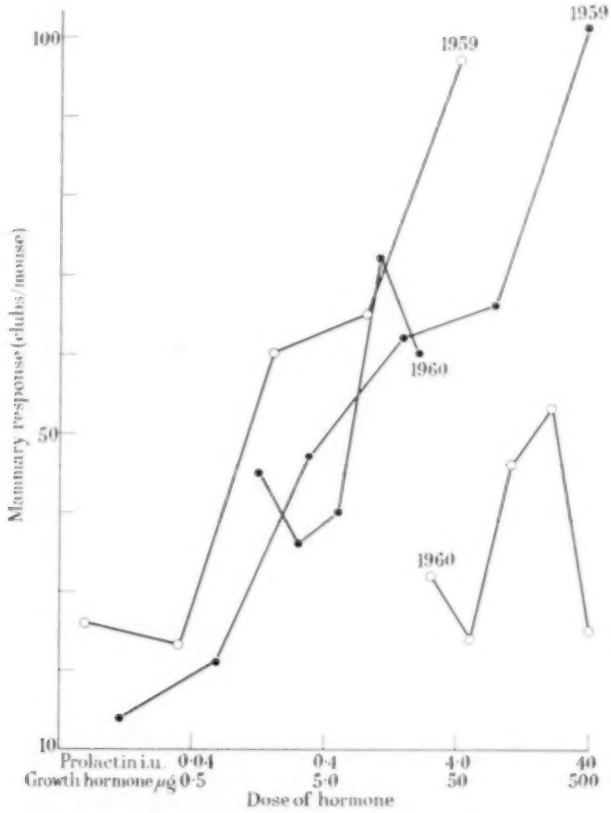


FIG. 1.—Dose : response lines for prolactin (●—●) and growth hormone (○—○) in 1959 and 1960.

as sufficient confirmation of the more convincing evidence previously obtained (Hadfield, 1957 ; Hadfield and Young, 1958). It seems that the activity in urine must be concentrated if it is to be demonstrated with any regularity. The dose : response relations for prolactin were very similar to those obtained in a previous study (Young and Fraser, 1960) using the original steroid dosage but the two sets of results with growth hormone did not agree so well (Fig. 1). It may be noticed that both 1959 graphs flatten off at a count of about 50–70 clubs per mouse and there has been much evidence of a similar plateau in the present experiments with

prolactin (see Appendix). This may indicate that the mammary response is biphasic.

Despite simplification of the injection procedure, the modified club-count assay described in the Appendix is laborious and the variability of the mammary response from mouse to mouse severely limits the accuracy with which mammotrophic activity can be measured. Taken over several experiments the mean square error of the mammary response is about 1200, and the slope of the dose : response curve 20. If the strength of the test preparation is the same as the standard, the error of the log potency ratio (M), which is equivalent to the percentage error of the actual potency ratio, is roughly

$$\text{S.E. (M)} = \frac{s}{b}\sqrt{\frac{2}{N}}$$

where s^2 is the mean square error of the response, b the slope and N is the number of animals. Inserting the above values for s and b and correcting to natural logs it is found that about 120 mice are needed to give 25 per cent accuracy in the potency ratio. On the whole this is an optimistic estimate, and if the unknown preparation differed considerably from the standard, the accuracy would be much lower. On the other hand, the mean square error in some cases may be as low as 600. However there is little doubt that consistently reliable results could only be obtained by using much larger numbers of animals than is feasible in practice.

It may be that treatment with other hormones concerned in mammary development in addition to oestrone and progesterone would increase the sensitivity of the hypophysectomized mice or reduce the variability of the response. On the other hand, such a procedure might offset one of the advantages of the biological method in studies of mammary cancer ; its measurement of an effective activity that may be the resultant of the action of several hormones. Only further studies can decide this question or whether an alternative and more practicable method of assay can be devised.

SUMMARY

Mammotrophic activity was assessed by injecting material to be tested into hypophysectomized weanling male mice of the A2G strain that were being injected at the same time with oestrone and progesterone and counting the increase in the number of swollen end-buds (clubs) of the mammary ducts.

Omitting samples that killed all, or all but one, of the test animals, activity was demonstrated in 3 out of 11 samples of urine, and in all of 3 samples of serum, from normal young women. Six out of 7 samples of serum from pregnant women were also active. No activity was demonstrable in 4 samples of urine from women past the menopause.

Some simplification of the method of testing was effected without reducing the variability of the response. This variability renders the method unsuitable for quantitative assays at present.

We are very grateful to the Endocrine Study Section, U.S.A. National Institutes of Health for generous gifts of prolactin and growth hormone, to Professor N. F. Morris, Charing Cross Hospital Medical School, for supplying the sera from pregnant women, and to Mr. John Gilbert and Mr. Michael Hart for much painstaking and assiduous technical assistance.

REFERENCES

HADFIELD, G.—(1957) *Lancet*, i, 1058.
Idem AND YOUNG, STRETTON—(1956*a*) *Brit. J. Cancer*, **10**, 145.—(1956*b*) *Ibid.*, **10**, 324.—(1958) *Lancet*, i, 568.
LYONS, W. M.—(1958) *Proc. Roy. Soc.* B, **149**, 303.
NANDI, S.—(1959) *Univ. Calif. Publ. Zool.*, **65**, 10.
READ, C. H. AND BRYAN, G. T.—(1960) *Ciba Found. Coll. Endocr.*, **13**, 68.
SIMKIN, B. AND GOODART, D.—(1960) *J. clin. Endocr.*, **20**, 1095.
YOUNG, STRETTON—(1957) *Brit. J. Cancer*, **11**, 116.
Idem AND FRASER, L. E.—(1960) *Ibid.*, **14**, 285.

APPENDIX

Attempted Improvement of the Assay

Steroid dosage

The doses of oestrone and progesterone (1 μg. and 5 mg.) used routinely seemed unnecessarily large. Reduction of the total doses to 0·1 μg. of oestrone and 1 mg. of progesterone did not influence the club count produced in response to 5 i.u. of prolactin. The same club count was also induced by 1 i.u. of prolactin suggesting that a plateau had been attained in the dose : response curve (Table III, Experiment A).

Steroid solvents

If lower doses of the two steroids were to be used then it should be possible to substitute a more convenient solvent than arachis oil, which is inconvenient to handle, persists at the injection site, and often interferes with the clean dissection of the mammary glands from the pelt.

Fifty per cent aqueous propylene glycol proved an unsatisfactory solvent. Responses were usually reduced in comparison with tests using arachis-oil solutions and the dose given (0·1 ml. twice daily) was on the borderline of toxicity for larger

TABLE III.—*Modification of Assay Procedure*

Experiment	Steroid solvent	Progesterone: Dose (mg.)	Progesterone: Number of injections	Oestrone (total dose μg.)	Prolactin (total dose i.u.)	Number of mice	Club count ± s.e.$_m$
A	Arachis oil	5·0	10	1·0	5·0	30	64 ± 6
		1·0	10	0·1	5·0	30	72 ± 6
		1·0	10	0·1	1·0	27	68 ± 5
B	Water	1·0	1	0·1	5·0	10	78 ± 10
		1·0	10	0·1	5·0	8	71 ± 8
		2·0	1	0·1	5·0	10	80 ± 10
		2·0	10	0·1	5·0	10	71 ± 2
C	Water	1·0	10	0·1†	5·0	13	61 ± 9
		1·0	10	0·1*	5·0	18	57 ± 6
		2·0	10	0·1†	5·0	8	66 ± 9
		2·0	10	0·1*	5·0	10	71 ± 2

† Oestrone and progesterone given together as mixed solution.
* Oestrone and prolactin given together as mixed solution.

volumes or increased concentration of the solution killed some, or all, of the mice injected.

The use of aqueous solutions or suspensions, however, had several advantages. An aqueous suspension of progesterone given as a single subcutaneous injection, was as effective as the same dose given in 10 injections (Table III, Experiments A and B) and aqueous solutions of oestrone could be mixed with the prolactin solution and injected in combination without affecting the response (Table III, Experiment C). This procedure not only reduced the labour of multiple injections but also eased the dissection of the pelts and was therefore adopted in all subsequent tests. With this simpler procedure it was possible to undertake a factorial experiment examining the change in response with variations in dosage of all three hormones.

Factorial experiment on hormone dosage

A 3 × 3 × 4 factorial experiment was set up with 3 doses of oestrone (0·05, 0·1 and 0·2 μg.), 3 doses of progesterone (0·5, 1 and 2 mg.) and 4 doses of prolactin (0, 0·5, 1 and 2 i.u.). There were originally 4 mice in each of the 27 groups receiving prolactin and 2 mice in each of the 9 groups receiving no prolactin. Since the different steroid doses had little, if any, effect on the response to prolactin, no elaborate statistical formulation need be given. In Table IV the full data are arranged with reference to prolactin dosage, first without regard to steroid dosage and then with regard to oestrone dosage and progesterone dosage separately. Clearly prolactin dosage within the range 0·5–2·0 i.u. had little if any effect on response and this is true whatever the steroid dosage within the range of 0·05–0·2 μg. of oestrone and 0·5–2 mg. of progesterone.

TABLE IV.—*Club Counts ± s.e.$_m$ in Factorial Experiment on Steroid Dosage*

	Dose of prolactin in i.u. (total number of mice)			
Analysis	0·0 (16)	0·5 (34)	1·0 (33)	2·0 (32)
All mice . .	10 ± 2	46 ± 4	51 ± 6	56 ± 5
Oestrone				
0·05 μg. .	12 ± 6	50 ± 8	42 ± 7	61 ± 9
0·10 μg. .	8 ± 4	47 ± 6	61 ± 11	51 ± 10
0·20 μg. .	10 ± 2	42 ± 8	50 ± 9	54 ± 9
Progesterone				
0·5 mg. .	13 ± 5	42 ± 8	36 ± 4	51 ± 9
1·0 mg. .	10 ± 5	46 ± 6	64 ± 16	60 ± 9
2·0 mg. .	8 ± 5	52 ± 8	55 ± 10	56 ± 9

Dose : response relation for prolactin and growth hormone

Since the variation in steroid dosages used in the previous experiment had no significant effect on the results, the median doses (1 mg. of progesterone suspension and 10 × 0·01 μg. of oestrone in aqueous solution) were used in assays of the mammotrophic activity of prolactin and growth hormone. The results (Table V) show clearly how individual variation in response could vitiate conclusions based on small groups of animals or small differences in dose. With 10 mice per group,

TABLE V.—*Responses to Prolactin or Growth Hormone given with 1 mg. Progesterone and 0·1 μg. Oestrone*

Prolactin		Growth hormone	
Dose (i.u.)	Club count ± s.e.$_m$	Dose (mg.)	Club count ± s.e.$_m$
0	18 ± 4	0	7 ± 4
0·12	45 ± 5	0·03	32 ± 10
0·25	36 ± 9	0·06	24 ± 10
0·50	40 ± 10	0·12	46 ± 15
1·00	72 ± 10	0·25	53 ± 12
2·00	60 ± 8	0·50	25 ± 4

a lower average response can be elicited by a 4-fold increase in the dose of prolactin (compare the responses to 0·12 i.u. and 0·50 i.u.) and a reduction in response on doubling the dose is common (cf. 0·12–0·25 i.u., 1·0–2·0 i.u.). Despite this variation a significant regression line is achieved ($b = 22 \pm 8{\cdot}8$, $t = 2{\cdot}5$, 12 D.F., $P < 0{\cdot}05$). With growth hormone, however, a very low average response to the highest dose made the regression line insignificantly different from horizontal ($b = 9{\cdot}5 \pm 14{\cdot}7$, $t = 0{\cdot}65$, 29 D.F., $P < 0{\cdot}05$).

THE INDUCTION OF TUMOURS IN MICE BY INTRAVAGINAL APPLICATION OF CHEMICAL COMPOUNDS

E. BOYLAND, R. T. CHARLES AND N. F. C. GOWING

From the Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.3, and The Royal Marsden Hospital, London, S.W.3

Received for publication February 23, 1961

Many workers have induced carcinoma of the uterine cervix by application of carcinogenic compounds, thus Teutschländer (1926) induced carcinomata of the cervix in rats by application of coal tar. Glucksmann and Cherry (1958) induced sarcomata of the genital tract in rats receiving local application of 9, 10-dimethyl-1, 2-benzanthracene and oestradiol benzoate. Scarpelli and Haam (1960) reviewed the subject of experimental carcinoma of the uterine cervix and consider that oestrogens have a cocarcinogenic or promoting action in the production of tumours in the genital tract.

Tumours have, however, been induced in the genital tract of animals by preparations or substances which are not generally considered to be carcinogenic. Thus Pratt-Thomas, Heins, Latham, Dennis and McIver (1956) produced tumours in mice by intravaginal application of pooled human smegma, and Gardner (1959) induced tumours in BC strain mice by intravaginal application of a mixture of urea, adipic acid and carboxymethyl cellulose. In both these cases the induction period was over 60 weeks.

Although there is no clinical evidence that the chemical contraceptive preparations cause cancer of the cervix or uterus, the spermicides used in such preparations should be tested for toxicity. As carcinogenic agents are generally nuclear poisons, and as spermatozoa consist mainly of nucleus, one might expect good spermicides to be carcinogenic. One of the previously used spermicides, 8-hydroxyquinoline, induced tumours when implanted in the mouse bladder (Allen, Boyland, Dukes, Horning and Watson, 1957). Hoch-Ligeti (1957) described the induction of vaginal tunours by application of two contraceptive preparations (one of which contained 8-hydroxyquinoline) to rats maintained on a low protein diet.

With these observations in mind, tests were carried out with a series of spermicides which are used in contraceptive preparations. Control experiments with the medium alone and with the medium containing known carcinogenic compounds were carried out at the same time. The application of the medium polyethylene glycol (Carbowax 1000) alone, however, induced tumours in quite a high proportion of the treated mice. In the series treated with Carbowax containing 9,10-dimethyl-1,2-dibenzanthracene the tumours appeared much earlier than in the other groups. The results indicate that the repeated instillation of a chemically inert polymer into the vagina of the mouse induces carcinomata.

This action of the polymer may be related to the carcinogenic effect of other inert polymers, injected polyvinylpyrolidone (Hueper, 1956) or carboxymethylcellulose (Lehman, Lusky and Nelson, 1957) in rats.

EXPERIMENTAL

Substances under test were dissolved in polyethylene glycol (Carbowax 1000) and 0·1 ml. introduced twice weekly into the vagina of mice, using a syringe with a blunt needle. Each substance was administered to 20 mice, starting immediately after weaning. The mice were kept in metal boxes with originally 5 animals in each box. All mice were killed 18 months after the first treatment with the substances under test.

RESULTS

The results of the first experiment (Table I) show that although tumours occurred in most of the groups of mice, the highest incidence was generally in groups treated with substances which have induced cancer in other animals, i.e.

TABLE I.—*Incidence of Tumours of Cervix and Vagina in Stock Mice*

Material applied in Carbowax	Number of mice surviving		
	12 months	18 months	With carcinomata
Series 1			
Carbowax only	11	7	5
9,10-Dimethyl-1,2-benzanthracene (0·3 per cent)	10	0	9
2 bis(2-chloroethyl)amino-naphthalene (2 per cent)	9	4	6
Hexylresorcinol (1 per cent)	8	4	4
8-Hydroxyquinoline (1 per cent)	10	2	7
Ricinoleic acid (2 per cent)	10	7	4
Quinine sulphate (0·3 per cent)	8	6	4
Phenylmercuric acetate (0·5 per cent)	12	10	6
Toluquinone (0·3 per cent)	11	10	6
3-Hydroxyanthranilic acid (2 per cent)	12	6	2
Sodium *p* toluene sulphon chloramide (1 per cent)	14	11	3
Boric acid (2 per cent)	13	8	0
Stilboestrol (0·1 per cent)	9	5	1
Methyl *p* hydroxy benzoate (1 per cent)	16	8	0
Triton WR (2 per cent)	9	8	0
HOC 8* (2 per cent)	16	11	1

* HOC 8 is the code name of 1,8,15,22-Tetra (polyoxyethyleneoxy) 4,11,18,25-tetra (1,1,3,3,-tetramethylbutyl)-cyclotetra-*m*-benzylene.

9,10-dimethyl-1,2-benzanthracene (Bachman, Kennaway and Kennaway, 1938), 8-hydroxy quinoline (Allen *et al.*, 1957; Hoch-Ligeti, 1957). Tumours were induced by these two compounds and also by 2-bis(2-chloroethyl)aminonaphthalene much more quickly than by the other substances; all the mice treated with 9,10-dimethyl-1,2-benzanthracene were dead within 9 months of the start of the experiment.

The compounds which did not induce tumours (boric acid, methyl *p* hydroxy benzoate and the non-ionic surface active agent Triton WR) are all mild antiseptics. The low incidence in the stilboestrol group is noteworthy.

The definite incidence of tumours in the group of mice treated with Carbowax 1000 only might have been due to the low osmotic pressure of the polymer. For this reason, another series of experiments starting with 30 mice in each group, was carried out with carbowax containing sodium chloride as shown in Table II.

TABLE II.—*Incidence of Tumours of the Cervix and Vagina in Stock Mice*

Material applied in Carbowax	Number of mice surviving		
	12 months	18 months	With carcinomata
Carbowax alone	24	20	2
Sodium Chloride (0·8 per cent) .	22	14	5†
Sodium chloride (30 per cent) . .	18	14	8

† Including one tumour of anal gland.

The incidence of tumours was lower in the control series of this experiment, and was higher when the carbowax was made isotonic with salt and even higher with 30 per cent salt. This experiment shows that the carcinogenic action of carbowax is unlikely to be due to the effect of the low osmotic pressure.

PATHOLOGICAL OBSERVATIONS

All the tumours developing in this series of animals were carcinomas: no sarcomas were found.

Hyperplastic (? premalignant) changes.—Apart from definite tumour formation, various degrees of epithelial hyperplasia and hyperkeratosis were found in many animals. Blunt epithelial downgrowths often formed part of the hyperplastic process, and occasionally foci of nuclear irregularity and dyskeratosis were found (Fig. 1). There appeared to be all transitions between simple hyperplasia, carcinoma in situ and frank invasive cancer. The subepithelial tissues frequently displayed oedema and inflammatory-cell infiltration.

Tumour formation.—On naked-eye examination the vaginal neoplasms took the form of thick greyish plaques, papillary formations or bulky polypoid masses. The majority of the tumours clearly arose from a very extensive "field" of altered epithelium: the whole vaginal and cervical mucosa were often affected. Three different microscopic patterns could be distinguished, although it must be stressed that these were frequently associated within the same growth:

(1) "Basal cell" tumours, composed of compact groups of small darkly staining

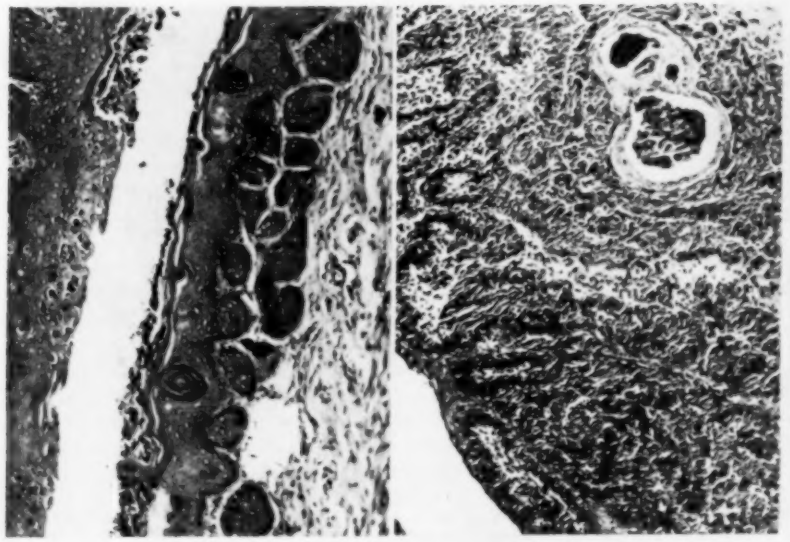
EXPLANATION OF PLATE

FIG. 1.—Longitudinal section through vaginal wall of a mouse implanted with HOC8. There is considerable hyperplasia and hyperkeratosis of the stratified squamous epithelium. Some dyskeratosis is seen in the epithelium in the upper part of the field. H. and E. ×112.

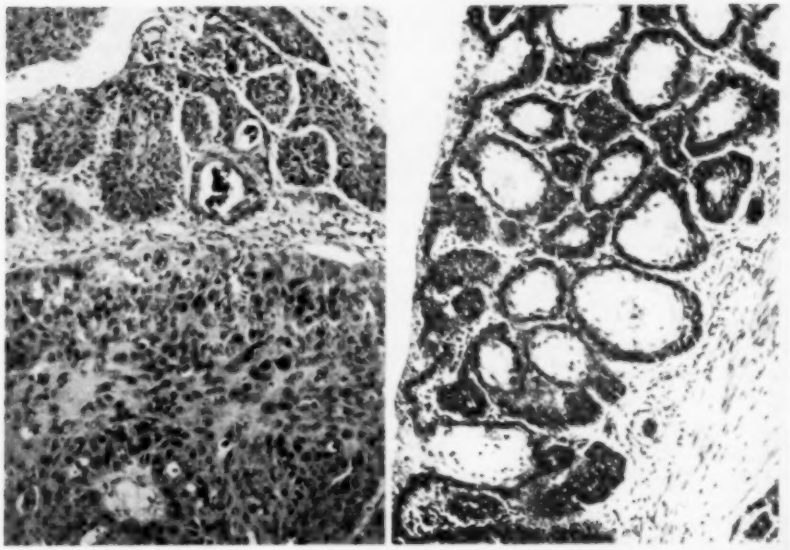
FIG. 2.—Mouse implanted with phenyl mercuric acetate. Infiltrating vaginal carcinoma composed of small darkly-staining "basal" cells. H. and E. ×112.

FIG. 3.—Mouse implanted with 8-hydroxy-quinoline. Poorly-differentiated squamous cell carcinoma infiltrating beneath hyperplastic epithelium of the vaginal wall. Nuclear pleomorphism, hyperchromasia and mitotic activity are all prominent. H. and E. ×112.

FIG. 4.—Mouse implanted with phenyl mercuric acetate. Section prepared from the edge of an infiltrating vaginal carcinoma showing "clear", mucin secreting cells at the centres of epithelial downgrowths. H. and E. ×112.



1 2



3 4

cells with hyperchromatic nuclei and scanty cytoplasm. There were no intercellular bridges and keratinisation was minimal (Fig. 2). These neoplasms somewhat resembled basal cell carcinomas of the skin but it is not suggested that they have any close biological kinship with them.

(2) Squamous cell carcinomas, displaying "prickle cell" formation and keratinisation. All grades of malignancy were found, from well differentiated slowly growing tumours to growths which showed nuclear pleomorphism and numerous mitoses (Fig. 3).

(3) Mucin secreting tumours. The mucous cells appeared to arise by differentiation of the proliferating basal cells as they approached the surface. Thus the fronds of a papillary neoplasm were sometimes covered by cells distended with basophilic mucin. On the other hand, where the proliferating cells were arranged as downgrowths, the mucous cell differentiation occurred towards the centres of the groups so that gland-like structures were found (Fig. 4). As might be expected, the tendency to mucin production was usually most marked in tumours occupying the cervix and upper vagina, but in some cases growths in the lower vagina showed extensive mucous cell metaplasia. Mucin secreting and squamous patterns were often combined ("muco-epidermoid" pattern).

DISCUSSION

The experiments indicate that cancer of the female genital tract in mice is induced by non-specific chemically inert materials. They leave open the question of the possible carcinogenic activity of spermicides used in contraceptive preparations. In order to test the possible carcinogenic activity of these spermicides, some medium must be used which does not itself induce tumours. Alternatively, it might be possible that if fewer applications had been made the incidence of cancer in mice treated with the carbowax alone would have been sufficiently low to make an assessment of carcinogenic activity possible.

Although the carbowax used in the experiments may have induced tumours, the material would probably not present a hazard to man. Carbowax is used in cosmetics and as a medium for medicaments.

SUMMARY

Mice were treated by intravaginal application of Carbowax 1000 alone, and containing known carcinogens, and with spermicides twice weekly for one year. Although tumours developed more quickly and in a higher proportion of mice treated with Carbowax containing 9,10-dimethyl-1,2-benzanthracene than in other groups, the incidence of tumours in the control series treated with Carbowax alone was so high that the possible carcinogenic activity of the spermicidal compounds could not be assessed.

We should like to thank Mr. R. O. Rees for help with these experiments. The research was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research : Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

ALLEN, M. J., BOYLAND, E., DUKES, C. E., HORNING, E. S. AND WATSON, J. G.—(1957) *Brit. J. Cancer*, **11**, 212.

BACHMAN, W. E., KENNAWAY, E. C. AND KENNAWAY, N. M.—(1938) *Yale J. Biol. Med.*, **11**, 97.

GARDNER, W. V.—(1959) *Cancer Res.*, **19**, 170.

GLUCKSMANN, A. AND CHERRY, C. P.—(1958) *Brit. J. Cancer*, **12**, 32.

HOCH-LIGETI, C.—(1957) *J. nat. Cancer Inst.*, **18**, 661.

HUEPER, W. C.—(1956) *Proc. Amer. Ass. Cancer Res.*, **2**, 120.

LEHMAN, A., LUSKY, M. C. AND NELSON, A. A.—(1957) *Fed. Proc.*, **16**, 318.

PRATT-THOMAS, H. R., HEINS, H. C., LATHAM, E., DENNIS, E. J. AND MCIVER, F.—(1956) *Cancer*, **9**, 671.

SCARPELLI, P. G. AND HAAM, EVON.—(1960) *Progr. exp. Tumor Res.* (Basle, Karger.) **1**, 179.

TEUTSCHLÄNDER, K.—(1926) *Z. Krebsforsch.*, **23**, 161.

CARCINOGENESIS IN THE PITUITARY DWARF MOUSE. THE RESPONSE TO DIMETHYLBENZANTHRACENE APPLIED TO THE SKIN

F. BIELSCHOWSKY AND MARIANNE BIELSCHOWSKY

From the Hugh Adam Cancer Research Department of the Medical School and the New Zealand Branch of the British Empire Cancer Campaign, University of Otago, Dunedin, New Zealand

Received for publication April 24, 1961

THE response to a carcinogen is determined not only by the nature and the quantity of the agent but also by genetic and epigenetic factors which influence tissue susceptibility. In mice the reaction to agents inducing lymphatic leukaemia depends on genetic constitution, endocrine status (Kaplan, Nagareda and Brown, 1954) and on the presence or absence of the thymus. In rats presence or absence of the adenohypophysis determines whether cancer of the liver will or will not develop after administration of azo dyes or of aminofluorene and related compounds. Generally it is not difficult to make a distinction between the role of genetic and of epigenetic factors in carcinogenesis, but in the case of the pituitary dwarf mouse it is less easy to differentiate between the two. Pituitary dwarfism is due to an inherited defect of the adenohypophysis and in consequence, through lack of trophic pituitary hormones, a complex hormonal deficiency is present in these animals, an epigenetic phenomenon. Although most of the secondary disorders affecting gonads, thyroids and adrenals can be rectified by substitution therapy, complete normalization of the female dwarf mouse has not been achieved.

It seems justifiable to assume that, on the whole, differences in response between dwarf mice and their normal sized litter mates are due to the underdevelopment of the anterior lobe of the pituitary present in the former. However, highly inbred stocks carrying the dwarf gene are not available and therefore even siblings must be heterozygous for many genes. In an attempt to clarify the respective roles of hormones and of genetic factors determining tissue susceptibility, we have reinvestigated the response of the skin to dwarfs to a topically applied carcinogenic hydrocarbon. The skin was chosen because of an apparent discrepancy between results obtained by Bickis, Estwick and Campbell (1956) in the dwarf and by Korteweg and Thomas (1939) in the hypophysectomized mouse.

MATERIALS AND METHODS

The material consists of 65 male or female pituitary dwarfs matched with 51 litter mates of normal phenotype and in most cases of the same sex which will be referred to as " controls ". In order to obtain greater uniformity in our stock, mice known by their breeding performance to be heterozygous for the dwarf gene were mated brother to sister or parent to offspring. In this way animals belonging to F_4–F_6 became available for Experiments II and III.

9,10-Dimethylbenzathracene (Roche, Basle) (DMBA) was dissolved in acetone (AR) at a concentration of 0·1 per cent. In Experiment I 0·03 ml. of this solution was given to the dwarfs and 0·1 ml. to the controls. In Experiment II the respective amounts were 0·05 and 0·1 ml. and in Experiment III both dwarfs and controls received 0·05 ml. The solution was dropped on to the interscapular region from a micropipette fitted with a plunger. In all experiments the carcinogen was applied 9 times at weekly intervals.

The animals were weighed and inspected each week and all changes observed in the skin were registered. Individual charts were made for 15 dwarfs and their controls belonging to Experiment II and for all animals of Experiment III. Only tumours persisting for at least a fortnight were recorded in the tables. Papillomas which regressed after being present for a minimum of 2 weeks and which did not reappear were classified as permanent regressions. The diagnosis of malignancy rests on histological evidence and is based on the invasion of the panniculus carnosus. Not every papilloma was sectioned, but all tumours which appeared to have grown into the dermis were examined histologically. The animals were killed when they were seriously ill or when a tumour had ulcerated. The experiments were started when the animals were 2 months old and terminated 40–43 weeks after the first application of the carcinogen. A complete autopsy was done on all animals.

RESULTS

At the start of the experiments the average weight of the dwarfs was 8 g., that of the controls 25 g. Losses due to the toxicity of DMBA were moderate, 5 dwarfs and 5 controls died before the appearance of the first experimental tumour. They have been omitted from Table I.

During the first month of the experiment it became obvious that the reaction to the carcinogen was not the same in the 2 groups. At the time when the 2nd and 3rd weekly doses were applied the skin of the dwarfs was hardly affected whereas most of the controls showed already in the 2nd week heavy loss of hair and often also some inflammation in the painted area. By the 4th week however, the degree of epilation had become similar in both groups. This difference in the rate of response was noted in all experiments, even in Experiment III where both groups received the same dose. Ulceration of the skin, varying from slight to severe, occurred in 17 of the normal sized mice of Experiments I and II and in 1 animal of Experiment III, but was never seen in a dwarf. Marked hyperkeratosis and focal accumulation of pigment were the two outstanding alterations of the skin of dwarfs prior to the appearance of neoplastic changes.

In the controls treated with 9 doses of 0·1 or 0·05 ml. of DMBA every mouse surviving for more than 7 weeks developed at least one tumour in the painted area, the maximum number of papillomas per animal being 32 with the larger and 7 with the smaller dose. Only in 60 per cent of the dwarfs neoplastic lesions were induced at the site where the hydrocarbon was applied. Tumour yield was not increased by raising the dose from 0·03 to 0·05 ml. Table I summarises the results obtained in the three experiments. The graph (Fig. 1) shows the cumulative percentage of tumour bearing animals in Experiments II and III and illustrates the delayed appearance of papillomas in the dwarfs.

When the amount of DMBA given to the controls exceeded that given to the dwarfs, the normal size litter mates invariably developed the greater number of

TABLE I.—*Total Incidence of Tumours (Experiments I–III)*

Experiment	Number of mice	Number of tumour-bearing mice	Total number of tumours*	Total number of malignancies
I	Controls 16	16	111	11
	Dwarfs 23	15	26	5
II	Controls 22	22	205	12
	Dwarfs 22	14	26	8
III	Controls 13	13	47	8
	Dwarfs 20	13	35	7

* Present at post mortem.

tumours. When both groups were treated in the same way this order was reversed in 3 of 13 pairs. In one case this may have been due to the early death of the normal sized animal but in the two others the controls survived the dwarfs by

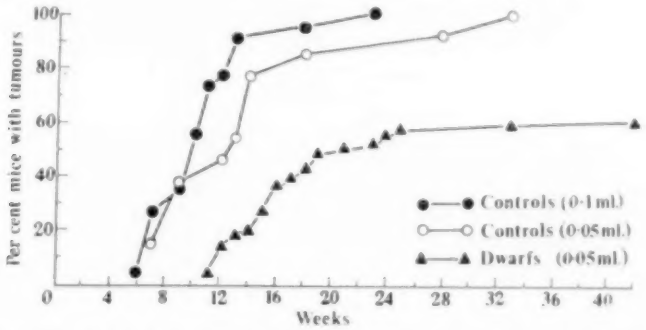


FIG. 1.—Latent period and percentage of tumour-bearing animals (Experiments II and III).

several months. Tables II and III give a detailed account of the responses of individual pairs. They show that the similarity in response of the respective groups is far greater than that of individual pairs. The most susceptible or resistant animals did not belong to the same litters.

In both groups a small percentage of tumours regressed and did not reappear during the time of observation. Carcinomas occurred in dwarfs as well as in controls in a similar percentage of tumour bearing animals. The time required to reach the state of malignancy varied from animal to animal, in a few dwarfs it was a matter of one or two weeks; in fact occasionally one was in doubt whether the neoplasm was not already invasive at the time it was discovered.

The material at our disposal did not allow systematic histological investigation of the pre-neoplastic changes induced by DMBA. However, in the animals which were autopsied during the first 3 months of the experiment, the same picture was seen in both groups, namely a patchy hyperplasia of the epidermis, loss of hair and sebacious glands, as well as condensation of collagen fibres and accumulation of mast cells in the dermis. The lower tumour yield in the dwarfs was certainly not due to lack of hyperplasia.

TABLE II.—*Comparison of Response of Controls and Dwarfs belonging to* 10 *Litters (from Experiment II)*

Litter	Sex C	Sex D	1st tumour (weeks) C	1st tumour (weeks) D	Total number of tumours C	Total number of tumours D	Permanent regressions C	Permanent regressions D	Number of Ca* C	Number of Ca* D	Duration (weeks) C	Duration (weeks) D
1	♂	♂	7	13	8	5	1	2	1	2	25	37
2	♂	♀	7	—	2	0	0	0	0	0	16	16
3	♀	♀	7	11	25	1	5	0	0	0	24	13
	♀	♀	9	—	22	0	2	0	1	0	17	24
4	♂	♂	7	11	20	2	3	0	1	1	26	14
	♀	♀	10	—	8	0	3	0	1	0	24	30
5	♂	♂	7	—	10	0	1	0	1	0	32	40
	♂	♂	10	—	11	0	3	0	1	0	36	43
6	♂	♂	9	15	2	1	0	0	0	1	22	26
	♂	—	12	—	12	—	0	—	0	—	27	—
7	♂	♂	10	12	8	2	0	0	1	0	26	22
	♀	♀	11	16	5	2	0	0	0	1	40	33
8	♂	♂	10	42	14	1	0	0	1	0	23	43
9	♂	♂	13	12	12	2	4	0	0	1	40	25
10	♂	♂	13	18	13	3	3	1	0	0	40	43

C = Controls. D = Dwarfs.
* Carcinoma.

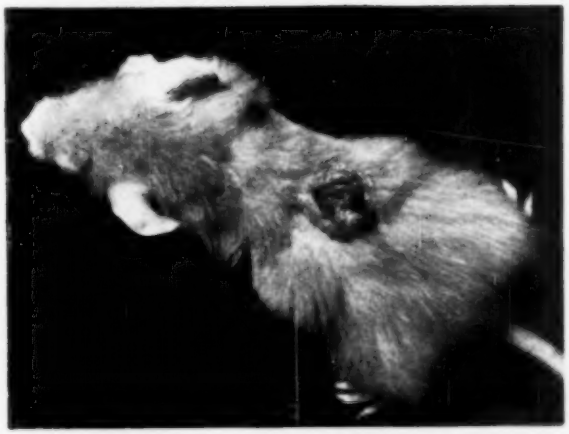
TABLE III.—*Comparison of Response of Controls and Dwarfs belonging to* 8 *Litters (from Experiment III)*

Litter	Sex C	Sex D	1st tumour (weeks) C	1st tumour (weeks) D	Total number of tumours C	Total number of tumours D	Permanent regressions C	Permanent regressions D	Number of Ca* C	Number of Ca* D	Duration (weeks) C	Duration (weeks) D
1	♀	♂	7	—	4	0	0	0	1	0	20	30
2	♂	♂	7	12	8	4	0	1	1	1	22	26
	♂	♂	14	14	3	2	0	0	1	0	20	25
	—	♂	—	16	—	6	—	1	—	0	—	33
3	♂	♂	9	19	3	3	0	0	1	1	22	26
4	♂	♂	9	—	4	0	0	0	1	0	41	41
	♂	♂	12	—	2	0	1	0	0	0	41	41
	♀	♀	13	23	3	1	0	0	1	1	41	27
	♀	♀	14	33	7	1	1	0	0	1	41	35
5	♀	♀	9	18	2	1	0	0	1	1	16	26
	♂	♀	18	—	5	0	0	0	0	0	40	41
6	♂	♂	14	21	7	2	0	0	1	0	36	22
7	♂	♂	28	13	1	2	1	0	0	0	41	24
	—	♂	—	—	—	0	—	0	—	0	—	27
8	♀	♀	33	15	1	5	0	0	0	2	41	38

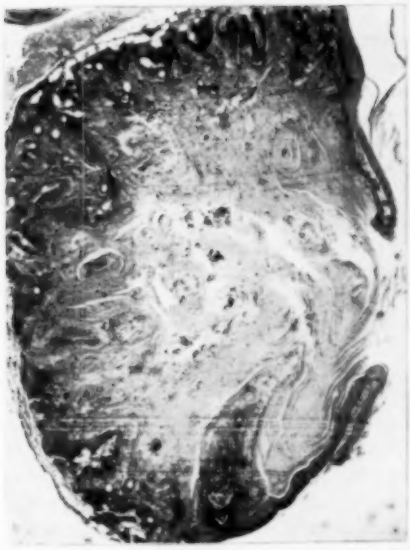
C = Controls. D = Dwarfs.
* Carcinoma.

EXPLANATION OF PLATE.

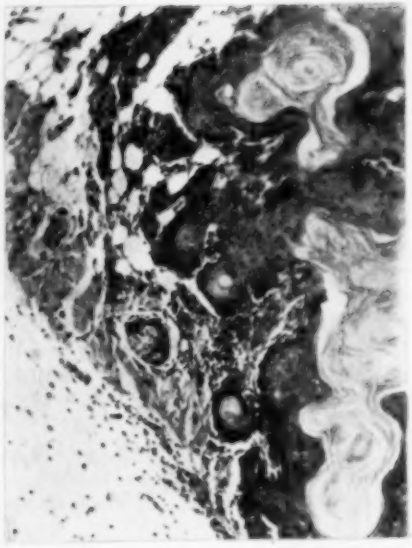
FIG. 2.—Dwarf with keratoacanthoma (Experiment II).
FIG. 3.—One of the 2 keratoacanthomatous lesions constituting the tumour seen in Fig. 2. H. & E. × 24.
FIG. 4.—Border of tumour seen in Fig. 3 showing beginning of invasion. H. & E. × 95.



2



3



4

The types of tumours arising in the treated area were also alike. In this connection it might be mentioned that in contrast to what occurs in man or rabbit only one keratoacanthoma regressed. Most showed a definite tendency for progression and became malignant (Fig. 2–4). Our experience with this type of tumour is in accordance with that of Della Porta, Terracini, Dammert and Shubik (1960).

A few neoplastic lesions of internal organs were discovered at autopsy. One dwarf had a haemangioma of the spleen and another a leukaemia. In the controls one reticulosis, one ovarian tumour, one adenoma of the lung and a polyp of the duodenum were found.

DISCUSSION

In the experiments presented the reaction of the skin to a topically applied carcinogen was investigated in animals the size of which differed considerably. The question arises what dosage should be used in such a comparative study. In the dwarfs amounts of 0·05 ml. wet a fairly large area of the back but in normal sized mice little more than the interscapular region. In view of the fact that the susceptibility of the skin decreases towards the root of the tail and towards the abdomen it was felt that areas of comparable anatomical rather than of absolute size ought to be the basis of comparison. Since, however, the quantity of DMBA administered influences also tumour yield, in the third experiment equal amounts were given to both groups. It is not feasible to administer more than 0·05 ml. to dwarfs and therefore it seemed better to reduce the volume given to the controls to the same level rather than to alter the concentration. This resulted in a considerable reduction in the number of tumours induced in the latter. In the first two experiments there was a remarkable difference in tumour yield between the two groups; in the third, although the trend for a greater number of papillomas was still discernible the difference between 47 tumours in 13 controls and 35 in 20 dwarfs is not any more statistically significant. The percentage of tumour bearing animals, however, remained the same as in Experiment II, namely 100 per cent in the controls and approximately 60 per cent in the dwarfs and there was no change in the period of latency, i.e. 6–7 weeks in the case of the phenotypically normal mice and 11 weeks in the case of the dwarfs. Once papillomas were present the chance that one of them would progress to malignancy was equal in both groups.

In principle our results agree with those of Korteweg and Thomas (1939) who compared the reaction of intact and of hypophysectomized mice to benzopyrene dropped on to the skin. In the former papillomas developed in all animals 44–100 days after treatment was started, but in the latter the first papilloma appeared at the 108th day. Only in 14 of 18 hypophysectomised animals surviving for more than 168 days benign tumours developed and in 10 of them they became malignant. These results were obtained in animals in which both groups were treated with the same dose of the carcinogen and in which the administration of benzopyrene was continued for up to 26 weeks. The mice were F_1 hybrids the father of which was invariably a dilute brown (Murray Little strain).

The results of Korteweg and Thomas (1939) and our own fit the hypothesis that hormones are the cause of the difference in the response of normal mice on the one hand and pituitary dwarfs or hypophysectomized mice on the other. They indicate also that the role of hormonal factors in the pathogenesis of neoplasms

derived from the epithelial cells of the skin is limited to the early phase of tumour development. Progression to invasive or metastasizing carcinomas seems independent of the endocrine status of the host.

SUMMARY

The susceptibility of the skin of pituitary dwarf mice to the action of DMBA was compared with that of their phenotypically normal litter mates.

Tumours developed in 100 per cent of the controls but only in 60 per cent of the dwarfs even when both groups were treated with equal amounts.

The latent period was always longer in the dwarfs.

This investigation was supported by a generous grant made to the Hugh Adam Department by the McLelland Trust which is gratefully acknowledged.

We should like to express our thanks to Mrs. E. Muir and Miss E. Johnson for valuable assistance.

REFERENCES

Bickis, I., Estwick, R. R. and Campbell, J. S.—(1956) *Cancer*, **9**, 763.

Della Porta G., Terracini, B., Dammert, K. and Shubik, P.—(1960) *J. nat. Cancer Inst.*, **25**, 573.

Kaplan, H. S., Nagareda, C. S. and Brown, M. B.—(1954) *Recent Progr. Hormone Res.*, **10**, 293.

Korteweg, R. and Thomas, F.—(1939) *Amer. J. Cancer*, **37**, 36.

INTRANASAL ADMINISTRATION OF 9,10-DIMETHYL-1,2-BENZANTHRACENE TO RATS: THE DEVELOPMENT OF BREAST AND LUNG TUMOURS

J. S. HOWELL

From the Department of Pathology, The Medical School, Birmingham 15.

Received for publication March 28, 1961

WHEN the carcinogen 9,10-dimethyl-1,2-benzanthracene dissolved in olive oil is applied to the skin of female rats at fortnightly intervals a high yield of breast tumours is obtained which are almost exclusively adenocarcinomata (Howell, 1959, 1960). It was pointed out that sometimes the skin became excoriated following this treatment, and that skin tumours as well as breast tumours were commonly produced. For this reason, and because it was desired to study the effect of ovariectomy on the growth of established tumours and their histological structure, and since surgical incisions are liable to become infected when superimposed on the skin changes caused by the carcinogen, alternative methods of administration were sought. In addition it was desired to show that DMB is also effective when given systemically. Accordingly the carcinogen was administered by intranasal instillation, a method which Orr (1943), using methylcholanthrene, has shown to be effective in producing breast tumours in IF mice.

MATERIALS AND METHODS

Twenty-nine virgin female and 13 male out-bred laboratory stock rats were used and at the start of treatment they were between 3 and 4 months old. They were kept in wire mesh cages, never more than 5 rats per cage and were given rat cubes (Heygate & Sons, known as the Thompson diet) and water *ad libitum*. A 1·6 per cent solution of DMB in olive oil was prepared, and before using was warmed to body temperature. At fortnightly intervals the animals were deeply anaesthetised with ether and carefully palpated for the detection of breast tumours. The rounded tip of the capillary of a Pasteur pipette filled with the carcinogenic solution was introduced deeply into one or other of the anterior nares. Under moderate pressure an average of 0·4 ml.–0·5 ml. or 6·4–8·0 mg. DMB were instilled over a period of about 30 seconds; immediately following this, the animal was held vertically, head uppermost, to facilitate drainage into the bronchi and to prevent oozing of oil from the nostrils over the face. Forceful inspiratory efforts usually followed instillation accompanied by a palpable "wet" chest; normal respiration was usually quickly re-established but was sometimes accompanied by sneezing with consequent expulsion of small quantities of the oil. Eight male rats were used as controls and were treated similarly to the experimental animals except that they received olive oil alone.

To determine whether the carcinogen was flowing into the respiratory passages, in certain animals that were deliberately killed, or in animals that died shortly after instillation, the respiratory passages, lungs, oesophagus and stomach were

carefully dissected, opened and examined under ultraviolet light. Although the oesophagus and stomach contained small quantities of the fluorescent carcinogen it was clear that most of it was present in the respiratory passages and lungs.

Treatment was continued until breast tumours developed or until the death of the animal. When a breast tumour was found the animal was usually killed, but in 6 animals with single breast tumours of approximately 1 cm. diameter, open biopsy, under ether anaesthesia removing about half the tumour was performed, at the same time both ovaries were removed, and further intranasal instillations of the carcinogen were stopped. This procedure was undertaken in order to assess the effect of ovariectomy on subsequent tumour growth and tumour histology.

Post mortem and histological methods

Blocks of tissue from all breast tumours were preserved, together with tissue from the right inguinal breast when this was possible. The lungs were removed in continuity with the larynx and trachea and were inflated with fixative and preserved for microscopic study. The ear ducts were always opened and inspected for the presence of tumours. The oesophagus and stomach were also routinely examined for tumours, but none was ever found. Tissue was preserved from any other organ showing gross pathological changes.

All tissue was fixed in 4 per cent formaldehyde-saline. Sections were cut and stained with Ehrlich's haematoxylin and eosin, Weigert's haematoxylin and Van Gieson and by Lawson's elastic stain. The Prussian-blue reaction was also performed on occasions.

RESULTS

Females

Of the 29 female rats, 5 died very early in the course of the experiment before the technique of intranasal instillation was fully mastered and death in all 5 was directly attributable to asphyxia following instillation. Survival in the remainder was good ; 24 survived for an average period of 5·8 months, receiving an average of 11·5 intranasal instillations. Eighteen of the 24 (75 per cent) developed breast tumours, and in 7 of the 18 more than one breast tumour was found ; the first breast tumour appeared after 4 months treatment. The 6 rats that had had breast tumours biopsied received an average of 10·5 instillations and survived for an average of 7·25 months, whereas those not biopsied received an average of 13·6 instillations and survived for an average period of 6·8 months.

Of the 6 rats submitted to biopsy and ovariectomy, one died 10 days post-operatively, 4 died between 4 and 8 weeks after the operation and the remaining animal survived for 4 months. Discarding the animal that died within 10 days, the rat that lived for 4 months when examined post mortem had no trace of tumour in any mammary gland. In 3 animals the portions of breast tumour left *in situ* grew progressively and in 2 of these, additional tumours in other breasts also developed. In the remaining animal there was apparent disappearance of the residual tumour for a period of one month, nothing being palpable in the biopsied breast, but during the second month the tumour reappeared and grew rapidly, ulcerating the skin necessitating killing the animal.

Tumours arising in ear ducts were present in 8 of the 24 rats, and sometimes these were bilateral. No skin tumours were found.

Males

One male rat died early in the experiment, death being attributable to asphyxia following administration of the carcinogen. The remaining 12 survived for an average period of 7 months receiving an average of 14 treatments. Although 3 animals were lost due to cannibalism they had not had palpable breast tumours prior to death and none of the remaining 9 which were examined post mortem had breast tumours. Two animals developed ear duct tumours.

Lungs

It was possible to examine the lungs of 33 rats that survived for long periods and of these, only 9 had macroscopically normal lungs and pleural cavities. Five with abnormal lungs had pleural adhesions and effusions sometimes blood stained. In 4 other animals, 3 females and 1 male pulmonary tumours were found. The earliest of these was observed in a female after treatment for 6·5 months, the other 3 were found after an average period of 8 months. The tumours were all squamous cell carcinoma the histology of which will be described later.

Pathology

Breast tumours

The gross and microscopic appearances of the breast tumours induced by DMB in the rat have been described in earlier publications (Howell, 1959, 1960); suffice it to state here that the breast tumours induced in the present experiments were all adenocarcinomata which in range of histological structure were similar to those produced by applying the carcinogen to the skin.

The biopsy specimens all showed typical adenocarcinomata with no unusual features. The tumours examined post mortem from the same animals showed no consistent changes. There was an impression that secretory activity was less, and in some the cells were rather plumper in appearance than in the biopsy specimens. The stroma tended to be greater in amount and in some tumours there was a definite scirrhous appearance which was not present in the biopsy specimen. In 2 tumours there was evidence of squamous metaplasia which was not present in the biopsy. Whether the increase in stroma and squamous metaplasia were the result of ovariectomy or of the biopsy procedure with possible superimposed infection is not clear.

Lungs

The commonest abnormality consisted of multiple bronchiectatic nodules of varying size containing yellowish-white cheesy material and sometimes frank pus. Areas of collapse and consolidation of lobes or portions of lobes, together with emphysematous changes were also observed. Although 4 animals had tumours in the lung it was not possible to detect these on gross examination except in one instance where the main bronchus to one lung appeared thickened and its lumen was filled with keratin-like material (Fig. 1).

Microscopic examination showed varying degrees of bronchiectasis with a marked chronic inflammatory reaction around the bronchi, maximal around the larger bronchi but extending the whole length of the bronchial tree to its terminal branches. The bronchial lumen frequently contained basophilic secretion ad-

mixed with polymorphs. The bronchial mucosa was frequently ulcerated and lined in places by vascular, fibroblastic tissue containing numerous foamy macrophages (Fig. 2). This granulation tissue occasionally formed polypoid masses projecting into the bronchial lumen, sometimes completely filling it (Fig. 3). Varying degrees of squamous metaplasia was very common in bronchi of all sizes, sometimes involving only a small sector of a bronchus, but frequently the entire mucosa was replaced by squamous epithelium accompanied by the formation of keratin filling the lumen (Fig. 4). This change was maximal in the smaller bronchi and was more marked than that seen in spontaneous bronchiectasis or in the control animals. In a few animals, in main bronchi, small lesions resembling hyperkeratotic squamous papillomata were present (Fig. 5). Other changes in the mucosa were also observed; in some animals there was proliferation of the basal cells (Fig. 6 and 7) in others there were localised proliferations of mucosal cells forming polypoid, sometimes papillomatous, projections growing into the lumen (Fig. 8 and 9). In some animals changes similar to carcinoma *in situ* were observed (Fig. 10, 11 and 12). In all these lesions mitotic activity of the epithelium was increased. Epidermatisation of bronchial glands was frequently found. The 4 animals with bronchial tumours all showed advanced bronchiectasis with marked squamous metaplasia. The tumours were all well differentiated keratinising squamous cell carcinomata infiltrating the adjacent lung tissue (Fig. 13). They were all found adjacent to a bronchus from which they presumably had their origin and in 2 instances the carcinoma appeared to be multifocal in that tumour involved widely separated bronchi. No metastases from these tumours were found.

The adjacent lung tissue showed various changes: areas of lipoid pneumonia were frequently encountered, as was interstitial pneumonia with thickening of alveolar walls and infiltration by chronic inflammatory cells. In some animals

EXPLANATION OF PLATES

FIG. 1.—Carcinoma of bronchus. The wall of the bronchus is thickened and the lumen is filled with keratin-like material. Note also the small subpleural nodules.

FIG. 2.—Ulceration of bronchial wall showing replacement with lipo-granulomatous tissue. The bronchial lumen contains pus cells. H and V.G. × 130.

FIG. 3.—Polypoid mass of lipo-granulomatous tissue projecting into lumen of bronchiole and almost filling it. H. and V.G. × 130.

FIG. 4.—Bronchiectasis and squamous metaplasia. The lumen is filled with keratin. Note surrounding inflammatory reaction. H. and V.G. × 75.

FIG. 5.—Wall of main bronchus showing squamous metaplasia and part of a hyperkeratotic squamous papilloma. H. and V.G. × 58.

FIG. 6.—Bronchial mucosa showing basal cell hyperplasia. H. and V.G. × 170.

FIG. 7.—Bronchial mucosa showing early basal cell hyperplasia. The overlying ciliated epithelium appears normal. H. and V.G. × 420.

FIG. 8.—Papilloma of mucosa of a large bronchus. H. and E. × 100.

FIG. 9.—Polypoid overgrowth of the mucosa of a large bronchus. There is marked chronic inflammatory cell infiltration of the polyp and the adjacent bronchial wall. H. and V.G. × 92.

FIG. 10.—Bronchial mucosa showing squamous metaplasia and carcinoma *in situ*. H. and E. × 310.

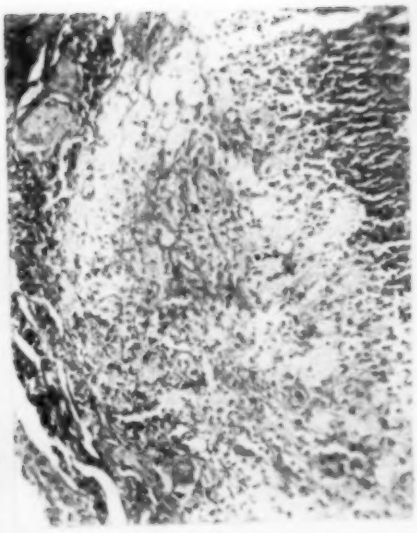
FIG. 11.—Carcinoma *in situ*. Similar to Fig. 10. H. and E. × 360.

FIG. 12.—Small bronchiole showing squamous metaplasia associated with carcinoma *in situ*. H. and E. × 225.

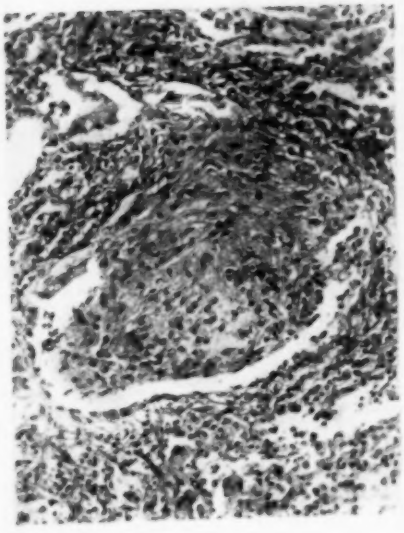
FIG. 13.—Keratinising squamous cell carcinoma. H. and E. × 60.

FIG. 14.—Squamous metaplasia of bronchial mucosa showing acanthosis and parakeratosis. H. and V.G. × 200.

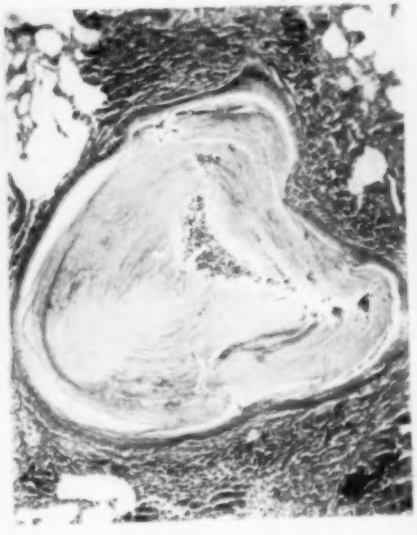
1



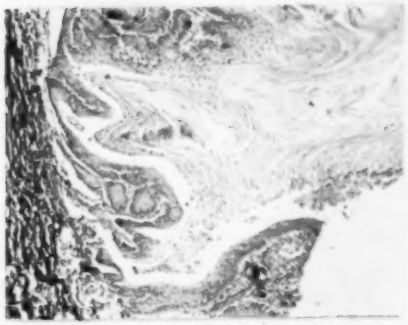
2



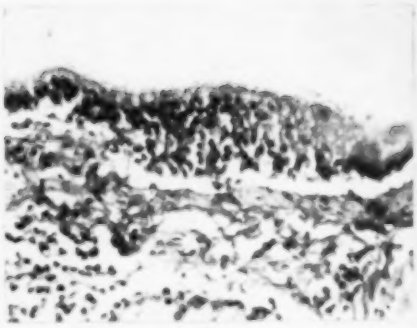
3



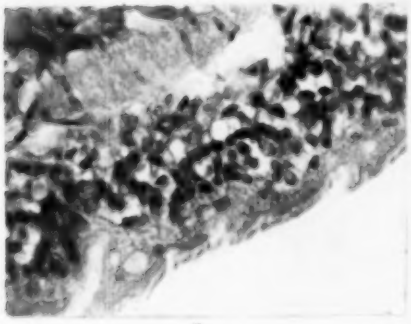
4



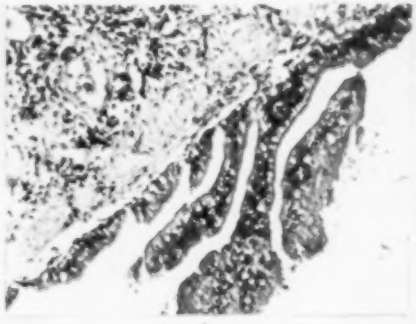
5



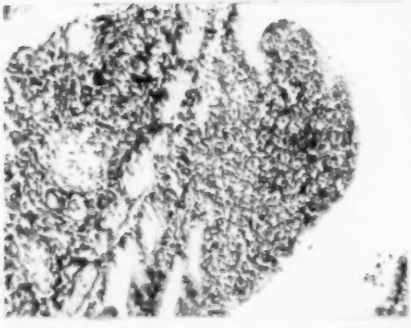
6



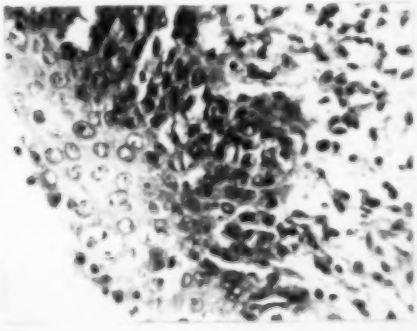
7



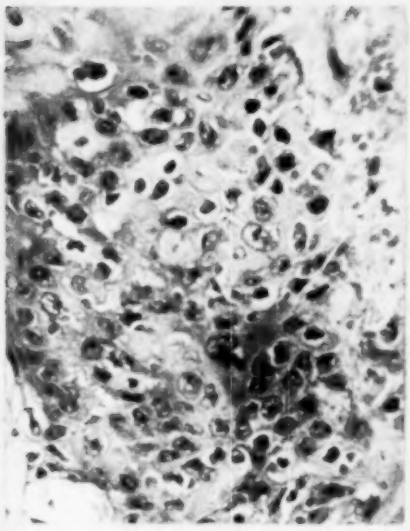
8



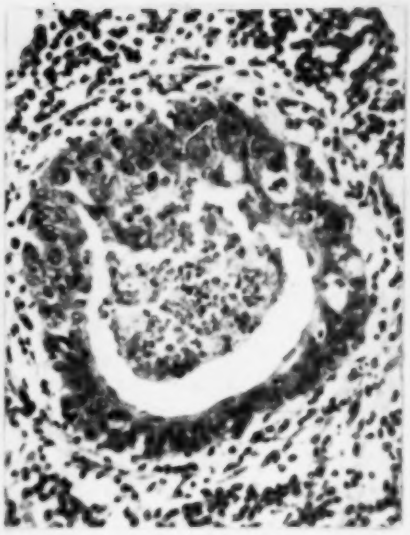
9



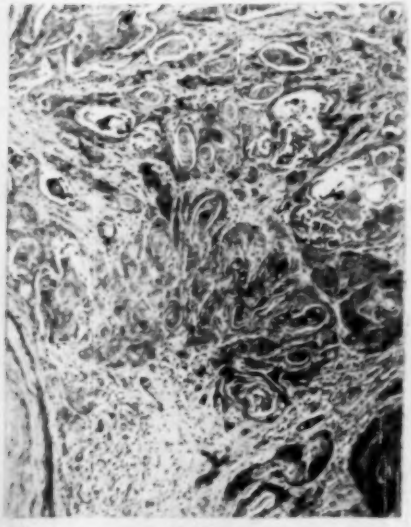
10



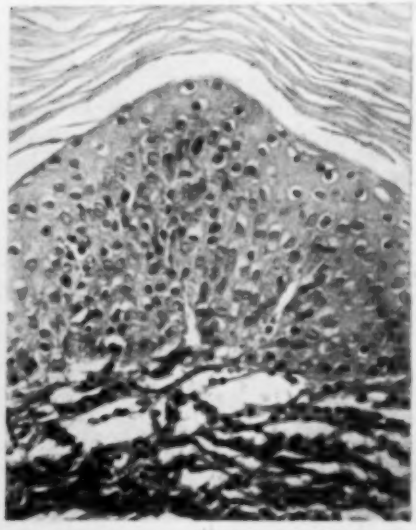
11



12



13



14

there was chronic collapse with superimposed infective changes, and in others frank pneumonia and abscess formation; most animals showed areas of emphysema. In many animals there was congestion and oedema, but these were probably terminal changes.

DISCUSSION

Breast tumours

These experiments show that DMB is effective in producing breast tumours following systemic administration and absorption. The breast tumour incidence (75 per cent) was almost the same as obtained when the carcinogen was applied to the skin (Howell, 1959), although the average induction time was approximately one month longer in the present experiments. This difference in average induction time can be explained by the lower total dose of carcinogen administered by the intranasal route, since it has previously been shown that the amount of carcinogen is of importance in determining average tumour induction time (Howell, 1959). The present experiments also confirm that normal male rats do not develop breast tumours in response to DMB, an observation previously made when the carcinogen was applied to the skin (Howell, 1960).

In view of the current interest in the hormonal control and dependence of breast cancer it is of interest that ovariectomy in one rat was followed by definite retardation in tumour growth over a period of one month, and in another animal the tumour disappeared completely. Whether this complete disappearance was the result of ovariectomy, or whether the residual tumour disappeared due to interference with blood supply and or infection following biopsy is not clear. However, ovariectomy had no effect in 3 out of 5 animals with established breast tumours and furthermore this procedure did not prevent the subsequent development of tumours in other breasts.

Huggins, Briziarelli and Sutton (1959) observed a reduction in growth rate of mammary tumours produced by methylcholanthrene in 7 out of 8 rats subjected to ovariectomy; the tumour in the remaining animal continued to grow. Similar reduction in growth rate was also observed following hypophysectomy, and administration of dihydrotestosterone. The greatest reduction occurred in the hypophysectomised group. These workers observed histological changes in those tumours which diminished in size following the various endocrine modifications. They consisted of atrophy and flattening of the epithelial cells lining the acini and absence of Pas-positive material in the acinar lumen. No constant changes were observed in breast tumour histology following ovariectomy in the present experiments. Tumour tissue was available from only one animal which had undergone regression and this showed less secretion than in the biopsy specimen and areas of squamous metaplasia, but the animal was killed whilst the tumour was growing rapidly. Obviously a much larger number of animals is required to assess ovarian hormone dependence of DMB-induced mammary tumours.

Lung tumours

In recent years there has been great interest in the problem of bronchogenic carcinoma, because of its increasing incidence and the statistical association between it and cigarette smoking. Numerous attempts have been made to con-

firm this association by experimental methods, but although some workers have obtained skin tumours in mice and rabbits by painting with tobacco smoke condensates (Wynder *et al.*, 1953), the production of bronchial carcinoma by the condensates has proved difficult. That this is not due to inability of small laboratory animals to develop lung carcinoma has been shown in numerous experiments using other substances. Perhaps the most consistent results have been obtained using various minerals, e.g. intratracheal insufflation of radioactive barium sulphate (Cember and Watson, 1958), and inhalation of radioactive cerium (Lisco and Finkel, 1949). Exposure to beryllium (Vorwald, 1952) has also resulted in bronchial cancer. More recently Hueper (1959) has shown that inhalation of nickel dust and intrapleural implantation of chromite ore roast are also capable of inducing lung tumours in rats and guinea-pigs.

Carcinogenic hydrocarbons have also been used. Probably the first high yield of experimentally produced lung tumours was obtained by Andervont (1937) implanting 1,2 : 5,6-dibenzanthracene impregnated threads through the chest wall of mice. Kuschner *et al.* (1956) used polycyclic hydrocarbons administered by inhalation, intratracheal injection, implantation of carcinogen-impregnated threads, and by direct introduction into bronchi of carcinogenic pellets. The first two methods failed to yield tumours, but they were obtained by the latter two methods. Blacklock (1957) was also able to obtain tumours by exposing the lung of rats and introducing 3, 4-benzpyrene, methylcholanthrene and tobacco smoke condensate.

It will be seen that many of the methods used for getting the carcinogen into contact with the bronchial epithelium have been complicated and bear little resemblance to the presumed route of contamination in the cigarette smoker. However recently Della Porta, Kolb and Shubik (1958) have produced squamous cell carcinomata in hamsters by repeated instillation into the trachea of a colloid suspension of DMB. This technique has the advantage of simplicity and is comparable to the method used in the present experiments.

The changes observed in the lungs in the present experiments consisted of bronchiectasis and its complications, mucosal abnormalities and frank squamous cell carcinomata. Bronchiectasis with associated squamous metaplasia, although a common disease in certain colonies, is uncommon in the out-bred rats used in these experiments, at least until they are between 2 and 3 years old. Even then the degree of bronchiectasis and squamous metaplasia occurring spontaneously is much less than that observed in these experimental animals. Furthermore, squamous cell carcinoma has never been observed spontaneously, either alone, or in association with bronchiectasis in these out-bred rats. Mucosal abnormalities of a type not seen in spontaneous bronchiectasis were common, consisting of basal cell hyperplasia, polypoid outgrowth of bronchial epithelium and occasionally lesions resembling carcinoma *in situ*. Squamous metaplasia was the rule rather than the exception, and it showed all degrees of differentiation, including acanthosis and parakeratosis ; keratin production was frequently very marked (Fig. 14). The bronchial tumours which were observed all occurred in association with squamous metaplasia of marked degree and this appeared to be an essential precancerous change. In control animals given olive oil alone survival was comparable to the DMB-treated animals. Mild bronchiectasis was found in the controls, presumably due to the bronchial obstruction caused by the olive oil, and was associated with moderate squamous metaplasia. However, the other

mucosal abnormalities described in the DMB-treated animals, and carcinoma were absent.

It is considered that had the DMB-treated animals survived longer, and had serial sections of the lungs been prepared more tumours would have been found, and it is concluded that this simple technique may offer possibilities for the experimental study of bronchogenic carcinoma.

SUMMARY

1. Experiments are described which show that 9,10-dimethyl-1,2-benzanthracene in olive oil is effective in producing breast carcinoma in female rats when administered by intranasal instillation. Male rats do not develop breast tumours.

2. No definite histological changes were observed in established breast tumours following ovariectomy. Although 2 of 5 rats showed regression of tumour growth, ovariectomy did not alter growth rate in the remaining 3 and did not prevent the development of additional breast tumours in 2 of these.

3. Changes in the lungs following intranasal instillation of the carcinogen are described, including the development of bronchiectasis and other mucosal abnormalities. In 4 animals squamous cell bronchial carcinoma were observed.

I am grateful to Professor J. W. Orr for helpful advice and criticism. My thanks are due to the Birmingham Branch of the British Empire Cancer Campaign and to the United Birmingham Hospitals Endowment Research Fund for financial support.

REFERENCES

Andervont, H. B.—(1937) *Publ. Hlth Rep., Wash.*, **52**, 1584.
Blacklock, J. W. S.—(1957) *Brit. J. Cancer*, **11**, 181.
Cember, H., and Watson, J. A.—(1958) *A.M.A. Arch. industr. Hlth*, **17**, 230.
Della Porta, G., Kolb, L. and Shubik, P.—(1958) *Cancer Res.*, **18**, 592.
Howell, J. S.—(1959) *Acta Un. int. Cancr.*, **15**, 163.—(1960) *Brit. J. Cancer*, **14**, 657.
Hueper, W. C.—(1959) *Acta Un. int. Cancr.*, **15**, 424.
Huggins, C., Briziarelli, G. and Sutton, H.—(1959) *J. exp. Med.*, **109**, 25.
Kuschner, M., Laskin, S., Cristofano, E. and Nelson, N.—(1956), *Proc. 3rd nat. Cancer Conf.*, p. 485.
Lisco, H. and Finkel, M. P.—(1949) *Fed. Proc.*, **8**, 360.
Orr, J. W.—(1943) *J. Path. Bact.*, **55**, 483.
Vorwald, A. J.—(1952) quoted by Cember, H. and Watson, J. A. (1958) *A.M.A. Arch. industr. Hlth*, **17**, 230.
Wynder, E. L., Graham, E. A., and Croninger, A. B.—(1953) *Cancer Res.*, **13**, 855.

OVARIAN AND MAMMARY TUMOURS IN INTACT C3Hb VIRGIN MICE FOLLOWING A LIMITED DOSE OF FOUR CARCINOGENIC CHEMICALS

C. BIANCIFIORI, G. M. BONSER AND F. CASCHERA

From the Division of Cancer Research, University of Study, Perugia, and the Department of Experimental Pathology and Cancer Research, University of Leeds

Received for publication March 13, 1961

It is now well established that mammary carcinomas and ovarian granulosa cell tumours can be induced, either separately or together, by means of chemical carcinogens in mice of various strains free of the milk factor. The IF strain has been most extensively studied and is highly susceptible. In regard to the breast, 9,10-dimethyl-1,2-benzanthracene (DMBA) and 20-methylcholanthrene (MC) are equally potent, 1,2:5,6-dibenzanthracene (DBA) induces an equivalent number of tumours at a late date and 3,4-benzopyrene (BP) is only weakly active (Bonser, 1958). In regard to the ovary, DMBA is by far the most potent chemical (Howell, Marchant and Orr, 1954; Marchant, 1957), BP and MC are possible weak carcinogens and DBA is inactive (Mody, 1960). 2-Anthramine was shown by Bielschowsky (1958*a*) to induce ovarian tumours in NZY mice, a strain where there is normally an excess of prolactin-secreting cells of the adenohypophysis (Bielschowsky, 1958*b*).

It was shown by Biancifiori, Bonser and Caschera (1959) that virgins and lobectomised females of the Balb/c strain did not develop mammary or ovarian tumours following the administration of MC but that a high yield of mammary cancer could be induced in mice made pseudopregnant artificially. As IF mice kept several in a cage undergo frequent spontaneous pseudopregnancy (Mody, 1959; Mühlbock and Boot, 1960) and BALB/c mice in the same conditions do not (Caschera, 1960), the common factor of pseudopregnancy was thought to explain the similar mammary response to methylcholanthrene administration. No ovarian tumours were induced in BALB/c mice (Table I).

It was thus thought desirable to study the action of chemical carcinogens in another strain free of the milk factor, namely C3Hb. The history of the Leeds and Perugian lines of this strain is as follows: H. B. Andervont obtained the C3H strain, having a high incidence of mammary cancer and later shown to carry the milk factor, from L. C. Strong in 1930. In 1942 (Andervont and Dunn, 1948) he eliminated the milk factor from a litter by removing the young from their mother before they ingested any of her milk and subsequently suckling them on C57 black females which had given birth to litters 24 hours previously. Three per cent of mammary tumours occurred in breeding females at an average age of 22 months but virgin females were not studied at this time.

Two litters of this line were sent in 1952 by H. B. Andervont to Leeds, where the mice have been continuously brother-sister mated. Later in the same year a litter was sent from Leeds to the Chester Beatty Research Institute, London.

TABLE I.—*Chemical Induction of Ovarian and Mammary Tumours in Intact Females*

Author and year	Strain or hybrid	Milk factor present	Pseudo-pregnancy	State of ovaries in untreated mice	Carcinogen	Mode of administration of carcinogen	Ovarian tumours: Mice treated	Ovarian tumours: %	Mammary tumours: Mice treated	Mammary tumours: %	Mean survival months
Marchant, 1957	IF	—*	+*	No tumours*	DMBA	In oil on skin	43	60	43	77	6·7
					MC	" " " " "	19	5	19	74	10·0
	IF × A				DMBA	" " " " "	35	40	35	80	6·8
					MC	" " " " "	21	5	21	62	13·5
	IF × C57B1	—*			DMBA	" " " " "	10	70	10	50	6·9
					MC	" " " " "	4	50	4	25	12·2
	A				DMBA	" " " " "	14	0	14	7	6·8
	C3H	+*			"	" " " " "	4	0	4	50	7·4
	C57B1	—*			"	" " " " "	27	11	27	0	9·3
	Outbred albino				"	" " " " "	22	18	22	18	6·6
Mody, 1960	IF	—	+	No tumours	DMBA	" " " " "	60	20			6·8§
					BP	" " " " "	53	8			7·3
					MC	" " " " "	37	0			5·1
					DBA	" " " " "	60	0			7·7
Bonser, 1958	IF	—	+*	No tumours*	DMBA	" " " " "			39	41	13·0
					BP	" " " " "			26	15	15·0
					MC	" " " " "			39	44	11·7
					DBA	" " " " "			21	43	15·0
Biancifiori, Bonser and Caschera, 1959	Balb/c	—	Absent in virgins	No tumours*	MC	" " " " "	36	0	36	0	10·4
			Absent in lobecto-mised virgins	No tumours	MC	" " " " "	41	0	41	2	9·7
			+artificial		MC	" " " " "	32	0	32	44	6·7
Andervont and Dunn, 1950	dba (sub-line 212)†				MC	In benzene on skin (varying doses)			14	29	8·0
									23	74	12·0
									42	47	12·0
									39	31	14·0
Ditto, 1953	DBAf/2				MC	Oral in oil	108‡	1	44	32	9·0

* No specific statement in the publication but presumed to be so from other work.
† Renamed DBAf/2 in 1953.
‡ Some of these mice received stilboestrol pellets.
§ Mice deliberately killed at 8-week intervals.

who in turn gave a litter to the Division of Cancer Research, Perugia, in November, 1953, where brother-sister mating has also been continued. Thus the lines in Leeds and Perugia are of the same origin and have been continuously inbred since 1942.

Heston (1958) freed C3H mice, obtained from Andervont, of the milk factor by caesarean section at term and fostering of the young on C57 black mice and he termed the substrain C3Hf (Committee on Nomenclature, 1960). Thirty-eight per cent of the females of the first 5 generations which were intensively bred developed breast tumours at an average age of 20 months, which was attributed to the strong genetic influence and the intensive breeding. The higher incidence compared with that described by Andervont and Dunn (1948) was explained by differences in husbandry. Twenty-two per cent of a total of 911 breeding females of generations 6–25 developed breast tumours. Forty-one ovarian tumours (tubular adenomas, granulosa cell tumours and those with a mixture of all types) occurred in these 911 breeders.

Deringer (1959) obtained a line of agent-free C3H mice, namely C3He, by transferring fertilized ova from the oviduct of C3H mice to the uteri of C57 black mice. In 99 virgin descendants of these mice 4 mammary carcinomas and 20 granulosa cell tumours of the ovary were observed at an average age of 24 months. In 155 breeders the incidence of mammary tumours was 55 per cent and of ovarian tumours 21 per cent.

Boot and Mühlbock (1956) discussed the virgin mammary tumour incidence in various lines of C3H mice deprived of the milk factor but did not mention ovarian tumours. It becomes clear that various sublines of the agent-free C3H strain have a tendency to develop spontaneous mammary and ovarian tumours at a late age, the actual incidence varying in different laboratories. Thus the study of carcinogen-induced tumours in C3Hb mice is particularly interesting and to this end four carcinogens were employed in the present experiments.

MATERIAL AND METHODS

At Leeds.—Descendants of the litter of C3Hb mice received from H. B. Andervont and in the 22nd and 23rd generation of inbreeding were brother-sister mated until 1955, when the present experiments were begun. The mice were distributed at random between the experimental and control groups.

At Perugia.—The litter received from the Chester Beatty Research Institute was in the 28th generation of inbreeding. The mice of the present experiments were in the 51st inbred generation.

Treatment

All the mice were virgin females kept 4–5 in a cage. Treatment was started at 10–16 weeks of age.

(a) *At Leeds.*—Sixty-two mice received 0·2 c.c. of an 0·5 per cent solution of 20-methylcholanthrene in arachis oil by stomach tube once per week for 8 weeks, a total dose of 8 mg. per mouse.

(b) *At Perugia.*—The mice were divided into three groups: Sixty mice received 0·2 c.c. of an 0·5 per cent solution of 9,10-dimethyl-1,2-benzanthracene in almond oil by stomach tube once per week for 8 weeks, a total dose of 8 mg. per mouse.

Fifteen mice received a similar dose of 1,2:5,6-dibenzanthracene by the oral method described above and 15 received skin application once a week for eight weeks of 16 drops of an 0·5 per cent solution in almond oil. It was calculated that approximately 5 mg. of carcinogen was applied each time and some would certainly be ingested by licking.

Fifteen mice received 3,4-benzopyrene by the oral method and 15 by skin application as described above.

RESULTS

Mammary Tumours

Incidence.—One-third of the mice treated with DMBA developed carcinomas of the mammary gland at or before the 37th week after the start of treatment, five tumours appearing during the 11th week and four more before the 19th week (Table II). Half of the mice treated with MC developed mammary tumours, but

TABLE II.—*Number of Mice Bearing Mammary Tumours After Treatment*

Chemical	Number of mice at start	Weeks after start of treatment								Tumours	
		5–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89	Total	Per cent
DMBA	60	9/51	3/33	4/20	0/7	—	—	—	—	16/51	31·4
MC	36	0/35	0/33	3/31	1/26	10/24	5/13	0/4	0/2	19/35	54·3
DBA*	30	0/29	0/29	2P/29	0/26	2P/23	—	—	—	4/29	13·8
BP*	30	0/28	0/27	0/26	1P/22	0/5	—	—	—	1/28	3·6

Numerator = Number of tumours.
Denominator = Survivors at the beginning of the stated period.
P = Painted.
* = Equal numbers of mice received the carcinogen by painting and oral administration

the earliest to appear was at 31 weeks and 15 out of 19 mice did not develop tumours until the 50th to 67th weeks. There was thus a time lag of 15 to 20 weeks in the occurrence of MC compared with DMBA tumours. The incidence of mammary tumours following DBA and BP treatment was respectively 13·8 and 3·6 per cent. Multiple tumours did not occur in the latter two groups.

Morphology.—The majority of tumours were of either irregular tubular, papillary cystic or solid polygonal cell type (Table III). A few small regular tubular tumours resembling spontaneous ones associated with the milk factor and two of entirely squamous type were encountered. Excluding these, squamous metaplasia occurred in 18 out of 49 tubular or solid polygonal cell tumours.

Tumours of the Ovaries

When the experiments were started there was no information as to what effects carcinogenic chemicals would have on the ovaries of C3Hb mice. Therefore the animals in all the groups were allowed to survive as long as possible. As death occurred at different periods in each group, comparison of the carcinogenic activity and of the changes leading to tumour formation in the various groups is only partially possible but certain tentative conclusions can be drawn and from these further experiments can be planned.

In the early part of the experiment, the importance of examining both ovaries microscopically was not realised. Both ovaries were inspected and the one con-

TABLE III.—*Morphology of the Mammary Tumours*

Chemical	Weeks of treatment	Tumour type: Small regular tubular	Irregular tubular: Squamous metaplasia (+)	Irregular tubular: Squamous metaplasia (−)	Papillary cystic: Squamous metaplasia (+)	Papillary cystic: Squamous metaplasia (−)	Solid polygonal: Squamous metaplasia (+)	Solid polygonal: Squamous metaplasia (−)	Pure Squamous	Total number of tumours in tumour-bearing mice
DMBA	5–19	1	3	0	2	0	4	0	0	10/9
	20–29	0	0	1	1	1	0	0	0	3/3
	30–39	4	0	2	0	1	0	0	0	7/4
MC	30–39	0	2	0	0	0	0	0	1	3/3
	40–49	1	0	0	0	0	0	0	0	1/1
	50–59	0	1	3 + 1*	0	6	2	0	0	13/11
	60–65	0	0	3	0	4	2	0	0	9/4
DBA	34–54	0	0	1*	0	0	1	2	0	4/4
BP	48	0	0	0	0	0	0	0	1	1/1

* Carcinosarcoma.

TABLE IV.—*Size of Ovarian Granulosa Cell Tumours*

Chemical	Size (mm.)	Weeks after beginning of treatment 10–20	21–40	41–60	61–70
DMBA ...	< 1	—	5	—	—
	1–5	4	8	2	—
	6–10	3	—	—	—
	>10	—	6	—	—
MC . .	< 1	—	—	—	—
	1–5	—	—	4	1
	6–10	—	—	1	2
	>10	—	—	1	—

sidered most likely to contain a lesion was examined. Some microscopical tumours may, therefore, have been missed.

In 20 untreated virgin mice over 50 weeks of age three microscopical luteomas and one microscopical granulosa cell tumour were found (Fig. 1, 2 and 3). It is thus concluded that there is a tendency amongst these mice to develop ovarian tumours. For convenience of presentation luteomas and granulosa cell tumours have been recorded separately, although it is not impossible that they may represent phases of growth of the same tumour. Tumours greater than 1 mm. in diameter have been arbitrarily classed as macroscopical and those of lesser diameter as microscopical.

Luteomas

Incidence.—In the ovaries of mice treated with DBA and BP, all of which were dead at 54 weeks, a greater number of luteomas occurred than in control mice over 50 weeks of age (Fig. 1). When DMBA was the carcinogen, the number was comparable to that in control mice, though the tumours occurred at an earlier age. No luteomas were seen in the 18 MC-treated mice, which lived from 50–70 weeks. It must be pointed out however, that with DMBA and MC the granulosa cell tumours were large, no ovarian structure other than the tumour itself being seen. Also, in half the MC-treated mice only one ovary was examined microscopically.

Size.—No luteoma attained a diameter greater than 2 mm. apart from one tumour which measured 2·8 mm. and contained laminated thrombus in the centre. Nineteen measured less than 1 mm. We think it unlikely that these small nodules are autonomous at this stage but cannot assess whether they would grow and become fully developed tumours.

Number.—In 25 of 26 mice bearing luteomas the state of both ovaries was known: in 21 the tumours were unilateral and in 4 bilateral. In 21 ovaries they were solitary, two tumours being present in the remaining 8.

Structure.—The smallest luteomas have a well-defined fine connective tissue capsule and are composed of polygonal cells having a tendency to radial arrangement in single cell columns. The nuclei are large, vesicular and uneven in size; the cytoplasm is variable in amount, granular and eosinophilic (Fig. 3, 4 and 5). The centre of the nodule is usually occupied by vascular, loose connective tissue but on two occasions a dead ovum occupied the centre (Fig. 5). The larger tumours are also encapsulated but lose the radial cellular arrangement. They do not show follicular arrangement and no transition to granulosa cell type was seen.

Granulosa Cell Tumours

Incidence.—With the exception of the one microscopical tumour in a control mouse at 78 weeks of age, all the granulosa cell tumours occurred in treated mice, 28 out of 41 DMBA-treated mice (68 per cent), 9 out of 19 MC-treated mice (46 per cent) and 2 out of 26 (8 per cent) in DBA-treated (Fig. 2). The important

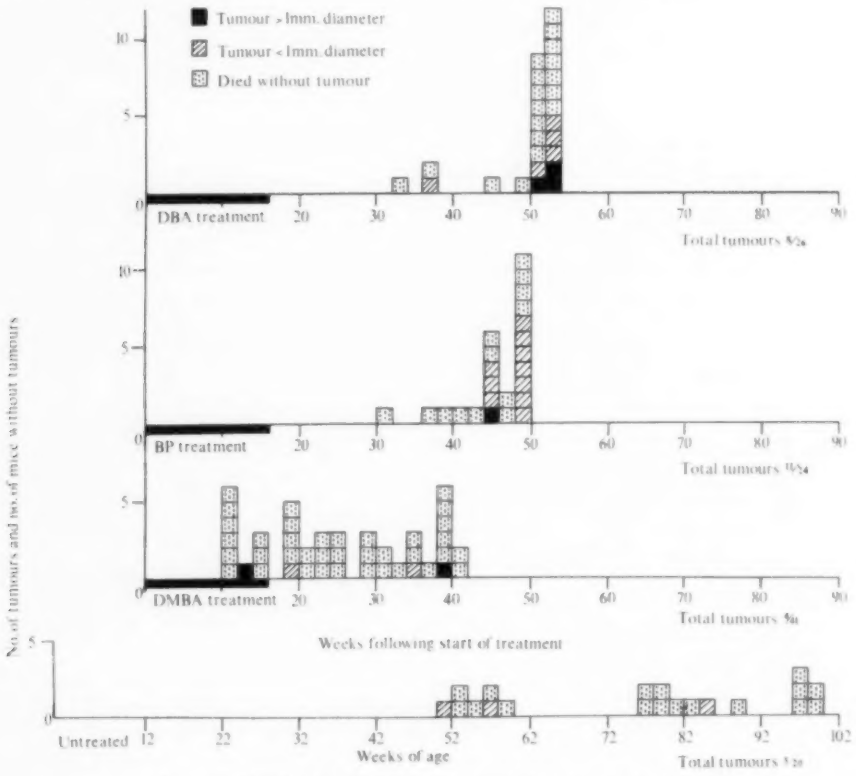


FIG. 1.—Distribution of luteomas in treated and control mice.

difference between the DMBA- and MC-treated groups is that tumours in the former occurred much earlier. Some were actually present before the application of the carcinogen had ceased and all had appeared before 42 weeks following the beginning of the treatment. The two tumours occurring in the DBA group were probably hastened in time of appearance but might well have occurred spontaneously.

Size.—The macroscopical tumours measured from 1·0–14 mm. in diameter, the average size in DMBA-treated mice being 5·4 mm. and in MC-treated mice 6·2 mm. (Table IV). All these were regarded as tumours which might be expected

to grow and persist. Some of the microscopical tumours measured less than 0·5 mm. in diameter, but even at this size they had a pseudofollicular structure and were probably autonomous.

Number.—Of 29 DMBA-treated mice in which the state of both ovaries was known, 13 had unilateral and 4 bilateral tumours. Of the latter, 3 mice bore macroscopical and 1 a microscopical tumour in one ovary, all combined with a microscopical tumour in the other ovary. Of 8 MC-treated mice in which the

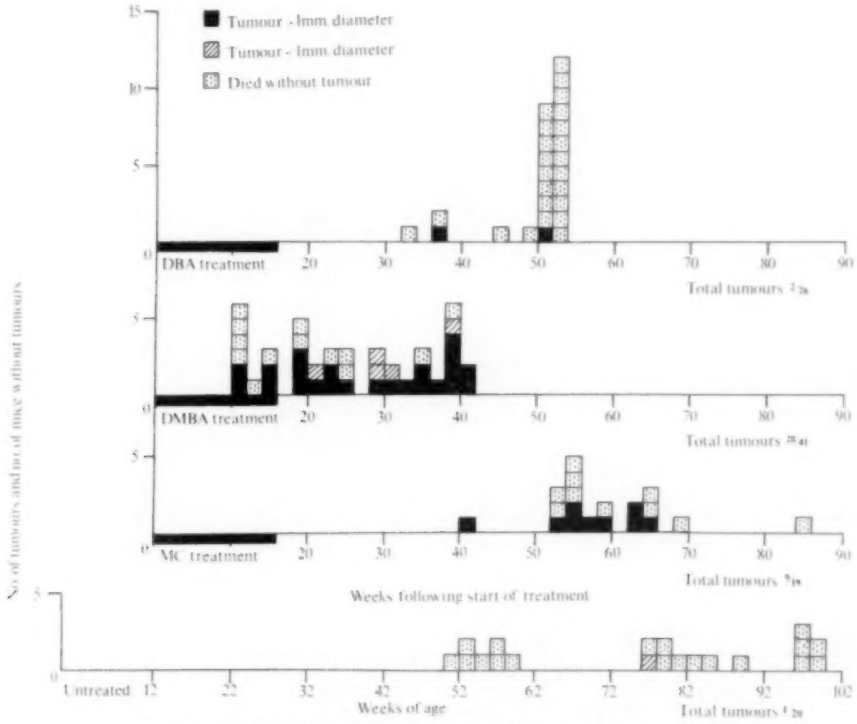


FIG. 2.—Distribution of granulosa cell tumours in treated and control mice.

state of both ovaries was known, 2 had large bilateral and one a large unilateral tumour. The tumours in DBA-treated and control mice were unilateral.

Granulosa cell tumours and luteomas did not occur together in the same mouse in one or both ovaries, except in one DBA-treated mouse dying at 38 weeks where two pseudofollicular granulosa cell tumours were present together with a microscopical luteoma in the same ovary.

Structure and size.—The structure of the fully-formed tumours has been amply described by Marchant (1957), and the present tumours of macroscopical size conformed to these descriptions. Without doubt they are true tumours. The difficulty lies in deciding whether some of the macroscopical "nodules" are

indeed tumours. The criteria of pseudofollicular structure and the presence of a good capsule (Fig. 6) seem to be the most helpful but it cannot be maintained that all the microscopical tumours would have persisted. No metastases were seen. A few tumours contained scattered areas where the cells appeared to be forming lutein but they were preponderantly granulosa cell tumours and did not appear to be derived from luteomas. Large granulosa cell tumours were observed in DMBA- and MC-treated mice (Table IV), but the tumours did not increase in size with increasing survival. The two tumours induced by DBA measured 2·0 and 1·1 mm. respectively. No untreated mice bore macroscopical tumours (Fig. 2).

Age Changes in Normal C3Hb Ovaries

Normal age changes in IF ovaries were described by Mody (1959). These consisted in a diminution in size of the ovary associated with progressive diminution in the number of follicles and corpora lutea. These structures were replaced by " dark staining cells " growing in from the germinal epithelium, anovular buds (also derived from the germinal epithelium), lipochrome-containing phagocytes, some cords of residual lutein cells and occasional fibrous or hyaline scars. The changes were usually well advanced by about 40 weeks of age.

Microscopical examination of both ovaries of all the control C3Hb mice in Fig. 1 revealed certain differences from IF mice. Between 50 and 60 weeks many dead and calcified ova are seen, small numbers of atretic and occasional ripening follicles persist and the main components of the ovary are intact or merged fully ripe corpora lutea (Fig. 7) and hilar cysts lined by a single layer of cuboidal epithelium and possibly of rete origin. At this stage pigment-containing phagocytes are conspicuously absent, indicating that the corpus luteum material is adequately removed. Between 78 and 100 weeks, dead and calcified ova and atretic follicles are still seen, although in diminishing numbers, intact corpora lutea have

---

EXPLANATION OF PLATES

FIG. 3.—Untreated virgin 85 weeks old. Small luteoma at top right. Wrinkled germinal epithelium, under which are a few dark staining cells. The main part of the ovary consists of bands of proliferated spindle thecal cells, some cut transversely, undergoing luteinisation. Small hilar cyst at bottom. ×75.

FIG. 4.—Virgin treated with BP for 49 weeks. Luteoma top right. The rest of the ovary is composed of germinal epithelium, merging corpora lutea and an atretic follicle. ×75.

FIG. 5.—Virgin treated with DMBA for 11 weeks. Small luteoma with dead ovum in centre. ×125

FIG. 6.—Virgin treated with MC for 56 weeks. Granulosa cell tumour of pseudofollicular pattern adjacent to telangiectatic area (part of which is seen top left). Rest of ovary shows thecal luteinisation. ×110.

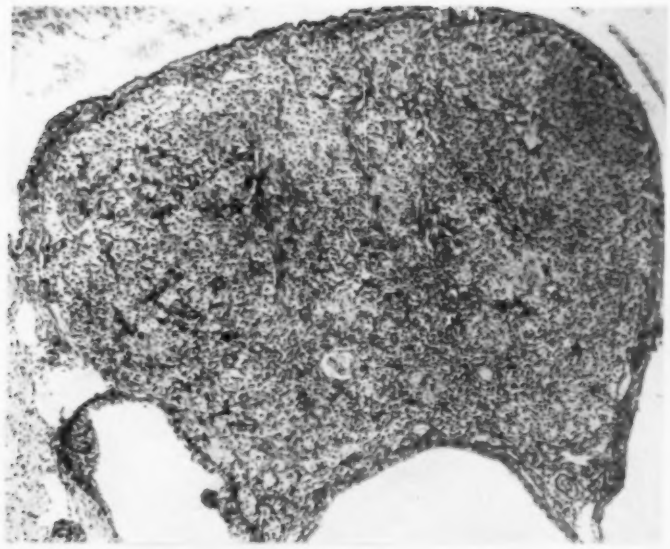
FIG. 7.—Untreated virgin 55 weeks old. Atretic follicles, dead ova, intact corpora lutea, dark staining cells and groups of lipochrome-containing phagocytes. ×120.

FIG. 8.—High power view of the same ovary as in Fig. 3 showing wrinkled dead ovum, and bands of spindle thecal cells cut longitudinally and transversely. ×140.

FIG. 9.—Untreated virgin 98 weeks old. Large hilar cyst on the right, atrophic remnant of the ovary to the left. Wrinkled germinal epithelium, dark staining cells, lipochrome-containing phagocytes and one dead ovum at bottom right. ×70.

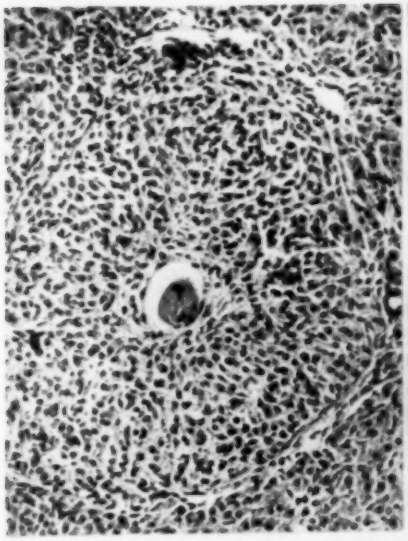
FIG. 10.—Virgin treated with DMBA for 23 weeks. Large numbers of dead and 2 calcified ova, between intact corpora lutea. ×80.

FIG. 11.—Virgin treated with MC for 55 weeks. Hilar cyst at top lined by columnar cells with vacuolated cytoplasm. Bands of luteinising thecal cells cut longitudinally and transversely. ×170.

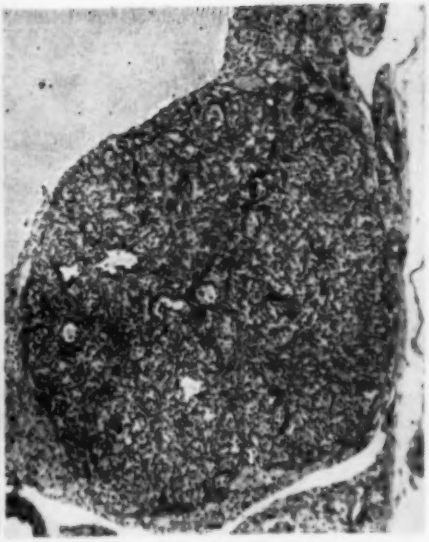


3

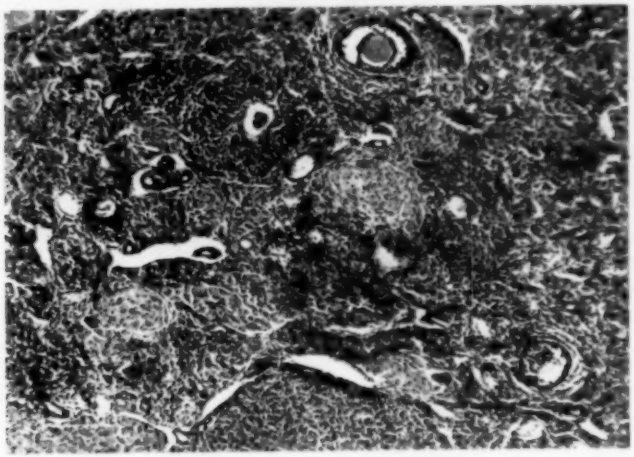
4



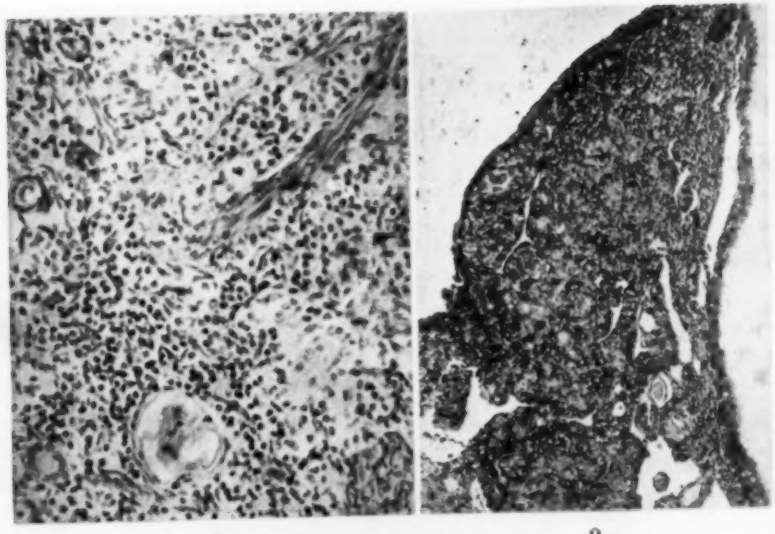
5



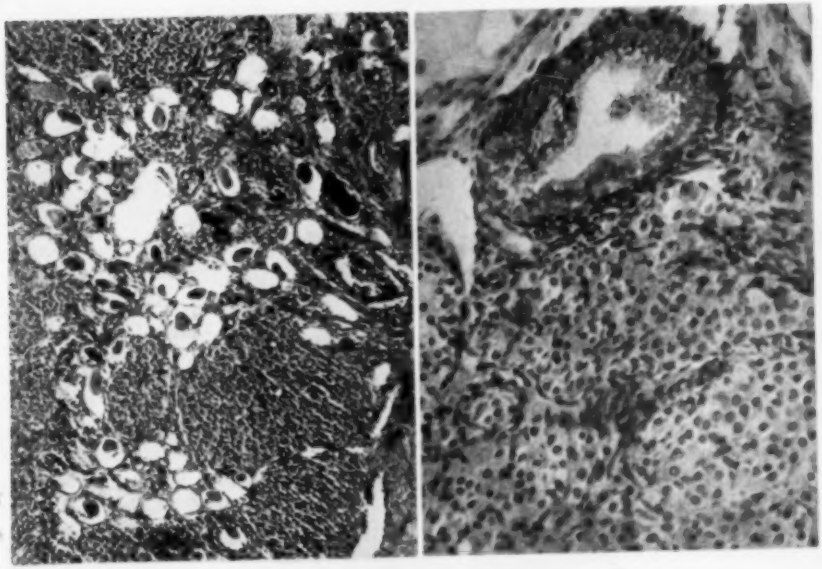
6



7



8 9



10 11

disappeared but lutein cell cords persist and pigment-containing phagocytes and dark staining cells are abundant. There is often theca cell proliferation, anovular buds and hilar cysts (Fig. 3, 8 and 9).

Non-tumourous Effects of Chemicals on C3Hb Ovaries

It is convenient to consider the effects of DBA and BP together, 50 and 32 ovaries respectively having been examined microscopically. Two main differences when compared with untreated ovaries of approximately the same age are seen (Table V): the presence of the small luteomas already described and the absence of hilar cysts. Apart from this the ovaries may be considered as having undergone normal age changes.

TABLE V.—*Ovarian Changes at Different Ages*

Chemical	Weeks following commencement of treatment	Dead and calcified ova	Atretic follicles	Persistence of corpora lutea	Anovular buds	Dark staining cells	Pigment containing phagocytes	Hilar cysts	Thecal proliferation
BP	32–50*	+	Few	+	0	0	0	0	0
DBA	33–54*	+	+	+	0	0	0	0	0
MC	42–85*	Few	0	0	Few to +	+	0 to + +	+	+
DMBA	11–41*	+ to few	Few	+ to few	0	0 to +	0 to +	0	0 to +
None	50–60†	+	+	+	0	0	0	+	0
	78–100†	+	Few	0	+	+	+	+	Little

* Add 12 weeks to give actual age.
† Weeks of age.

With MC and DMBA, however, the non-tumourous changes are different and will be described separately.

All the mice treated with DMBA were dead at 42 weeks after the beginning of treatment. As the large tumours occupied the whole ovary and as a few non-tumourous ovaries were not sectioned, the following description is based on 50 ovaries examined from 11 weeks onwards. Even at 11 weeks, dead and calcified ova are present, the remaining follicles are atretic and some of the corpora lutea are beginning to merge, though intact old corpora lutea are still present (Fig. 10). Anovular buds were absent throughout. At 19 weeks, thecal proliferation and luteinisation appears for the first time and becomes progressively more prominent as time proceeds, taking the place of the corpora lutea. Dead ova and small atretic follicles may still persist. Only occasionally are dark staining cells present in any numbers and hilar cysts were only seen in 3 of 50 ovaries. Thus the new feature of thecal proliferation and luteinisation is seen in ovaries treated with DMBA, from which dark staining cells, anovular buds and hilar cysts are absent (Fig. 11).

In Fig. 1 it is seen that two granulosa tumours arose at 11 and 12 weeks respectively, i.e. before thecal proliferation was observed. One of these occupied the whole ovary and in the other ovary no thecal proliferation was present alongside the small tumour in the section examined. This occurrence is not held to be sufficient to discount the idea that thecal proliferation is an essential precursor of tumour genesis.

From the 19 mice treated with MC, 17 ovaries not wholly occupied by tumour were examined at 53–85 weeks following the initiation of treatment. Dead and calcified ova are occasionally seen but atretic follicles and corpora lutea are entirely absent, their place being taken by thecal proliferation or, later, by pigment-containing phagocytes and dark-staining cells. Simple hilar cysts occur in nearly all the ovaries. Anovular buds increase in number as time proceeds but are never very numerous.

Other Tumours

Stomach.—Keratinising squamous papillomas or carcinomas occurred in all the groups. The highest incidence (26 per cent) was in MC-treated mice, but the tumours appeared late (42–85 weeks). Two-thirds were malignant. A similar incidence (24 per cent) occurred in DMBA-treated mice, but at an earlier age (11–40 weeks). In BP- and DBA-treated mice the incidence was 11 and 3 per cent respectively, the single tumour in a DBA mouse being a simple papilloma.

Liver.—Hepatomas occurred in two MC-treated mice at 56 and 63 weeks but none were observed in the other groups, where the animals died at an earlier age. In two control mice aged 98 and 99 weeks hepatomas were also present.

Pseudopregnancy

Fifteen normal C3Hb virgins were kept 5 in a cage. Daily vaginal smears were made for 60 consecutive days and the oestrous cycles were recorded (Table VI). Of 150 cycles, 21 were characteristic of pseudopregnancy, an incidence of 14 per cent. Mühlbock and Boot (1960) had previously found 12 per cent of pseudopregnancies in 332 cycles in C3Hf mice.

TABLE VI.—*Incidence of Pseudopregnancy in C3Hb and BALB/c Mice*

Strain	Number of mice examined	Number of cycles recorded	Number of pseudo-pregnancies	Per cent of pseudo-pregnancies
C3Hb	15	150	21	14
BALB/c (Caschera, 1960)	50	250	10	4

DISCUSSION

The response of virgins of the C3Hb strain to treatment with chemical carcinogens must be discussed in relation to what is known about the strain itself and what is known about the response of other strains to chemical carcinogens.

Comparison of spontaneous and chemically induced mammary and ovarian tumours in C3Hb mice

(a) *Mammary.*—No tumours occurred in the 20 control mice listed in Fig. 1 and 2 although 5 mice lived to the age of 98 weeks. Mammary tumours in Heston's (1958) virgin agent-free C3H mice occurred at an average age of 22 months and in Deringer's (1959) line at 20·7 months.

There is thus no doubt that the chemically induced tumours (Table II) occurred at an earlier period than the spontaneous ones. As the number of tumours induced with each chemical was so variable, ranging from 54·3 per cent following MC to

3·6 per cent following BP, it is concluded that these tumours were specifically induced by the chemicals. Morphologically the induced tumours had a greater variety of structure (Table III) than did spontaneous tumours. Only 20 of 51 tumours presented squamous metaplasia.

(b) *Ovarian.*—Among 20 untreated virgins (Fig. 1 and 2) a single microscopical granulosa cell tumour was observed at 76 weeks of age and 3 microscopical luteomas at 52, 56 and 86 weeks. Following chemical treatment the incidence of these types of tumour is greatly raised, the induction period is reduced and there is a dissociation of both these events according to the chemical used (Fig. 1 and 2). These facts lead to the conclusion that the chemicals specifically induce ovarian tumours. If the two types of tumour are considered separately it is clear that MC fails to induce luteomas whereas the other three chemicals are active in the order BP, DBA and DMBA. The latter chemical induces granulosa cell tumours early, whereas MC induces them late, and DBA and BP not at all. The size of the granulosa cell tumours is related to their yield (Table IV), the large tumours being found in mice treated with DMBA and MC.

Comparison of chemically induced mammary and ovarian tumours in different strains

(a) *Mammary.*—There is an interesting difference in the response of IF and C3Hb virgins to the four chemicals: in IF mice BP was the least potent chemical (15 per cent of tumours occurring after 36 weeks) whereas DMBA, MC and DBA each induced approximately 40 per cent of tumours. There was dissociation of induction time, DMBA and MC tumours occurring before 36 weeks and DBA tumours after (Bonser, 1958). In the present experiments, the range of tumour incidence was greater (MC 54, DMBA 31, DBA 14 and BP 4 per cent respectively). The DMBA tumours all occurred before 40 weeks but the majority of MC-induced tumours occurred after 40 weeks. It seems unlikely that the differing method of administration (painting in IF mice and oral in C3Hb mice) would account for this difference.

(b) *Ovarian.*—The important difference between IF and C3Hb mice in this respect is the high yield of granulosa cell tumours following MC treatment (9 large tumours in 19 mice). However, these tumours occurred after 50 weeks, whereas all the IF mice were dead at 48 weeks, and thus the time factor is probably of importance. The high early yield of tumours following DMBA treatment is comparable with that in IF mice.

Among the mice bearing granulosa cell tumours following treatment by MC two examples of large bilateral tumours were encountered, and one of a large tumour on one side and a microscopical tumour on the other. Large bilateral tumours were not encountered following DMBA treatment of IF mice (Marchant, 1959) or of C3Hb mice in this experiment.

(c) *Dissociation of mammary and ovarian tumours.*—Although both mammary and ovarian tumours might occur in the same mouse, as noted by Marchant (1959) and by Streeter (1960) in IF mice, there was no significant association between the occurrence of the two types of tumours.

(d) *Relation to pseudopregnancy.*—The incidence of pseudopregnancy in control virgin C3Hb females kept under the same conditions as the treated mice, is 14 per cent. As normal cycles last for about 5 days and each pseudopregnancy for about 12 days, the breast is under the influence of functioning corpora lutea for

25–30 per cent of the experimental period. In BALB/c mice only 4 per cent of the cycles are pseudopregnancies (Table VI) so that corpora lutea function only for about 12 per cent of the experimental period. Virgin BALB/c mice do not develop mammary carcinomas when treated with MC, in contrast with C3Hb and IF mice (44 per cent of pseudopregnancies (Mühlbock and Boot, 1960) which do. These findings support the work of Jull (1954) who found that oestrogen alone was not a sufficient hormonal stimulus to cause mammary tumours in castrated IF females when treated with a limited dose of the carcinogen MC but that the addition of progesterone resulted in an incidence of 82 per cent of mammary carcinomas.

Genesis of the ovarian tumours.—As normal C3Hb ovaries age they show two features not described by Mody (1959) in IF ovaries: calcification of the ova and hilar cysts, the latter probably derived from rete tubules. These structures are continuously present from 50–100 weeks, no information being available about the state of the ovary before this time. C3Hb ovaries age later but in the same manner as IF ovaries (Table V). When treated with chemicals the non-tumourous ovaries either age in normal fashion except for the lack of formation of hilar cysts (DBA and BP), or undergo a more rapid ageing process (MC and especially DMBA) associated with a proliferation of thecal cells. Mody thought that this was the essential precursor to the formation of tumour nodules but no direct evidence for this view has been derived from the present experiments.

Granulosa cell tumours were seen in all stages of development, from small nodules to large tumours. Occasionally luteinised areas were seen in these tumours and thus they might be called "mixed". The luteomas were all either microscopical or small; the largest measured 2·8 mm. in diameter (a diameter partly accounted for by the central haemorrhage) and the others 1·0–2·0 mm. As they occurred most frequently in DBA- and BP-treated mice, in which thecal proliferation is usually absent, it is unlikely that they arose in the same way as the granulosa cell tumours. From their early microscopical appearance and as the remnant of an ovum was found in the centre of two it seems possible that they arose in damaged follicles. No opinion can be expressed as to their autonomy.

SUMMARY

Virgin milk-agent-free C3H mice, named C3Hb and originally obtained from Andervont, were given a limited dose by oral administration* of 4 chemical carcinogens: DMBA, MC, DBA and BP.

C3H virgins when kept 5 in a cage undergo 14 per cent of pseudopregnancies.

Three luteomas and one granulosa cell ovarian tumour (all microscopical in size) occurred in 20 control virgins living to a greater age than the treated mice (Fig. 1 and 2). No mammary tumours occurred. Mammary and ovarian tumours occurred in breeding females of other lines of the strain maintained in the U.S.A.

C3Hb ovaries age in the same way but at a later date than do IF ovaries, with the exception of two characters: calcification of the ova and the formation of hilar cysts (Table V).

The ovarian tumours induced by DMBA (68 per cent) and MC (46 per cent) were of granulosa cell type, the former occurring earlier than the latter. Those induced by BP and DBA were luteomas.

* Half the DBA- and BP-treated groups were painted.

The granulosa cell tumours arose in areas of thecal proliferation after the follicles and corpora lutea had disappeared. The luteomas probably arose in damaged follicles. No evidence was obtained that granulosa cell tumours became luteomatous or *vice versa*.

Mammary tumours were induced by all four chemicals in the following descending percentage: DMBA (52·9), MC (31·4), DBA (13·8) and BP (3·6). DMBA tumours occurred earlier than all the others. The susceptibility of the mammary gland to chemical carcinogens is related to the fact that virgin mice undergo spontaneous pseudopregnancy.

C. Biancifiori and F. Caschera were supported by Grant C–3844 ((C1), National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Maryland, U.S.A.

REFERENCES

ANDERVONT, H. B. AND DUNN, THELMA B.—(1948) *J. nat. Cancer Inst.*, **8**, 227.—(1950) *Ibid.*, **10**, 895.—(1953) *Ibid.*, **14**, 329.

BIANCIFIORI, C., BONSER, G. M. AND CASCHERA, F.—(1959) *Brit. J. Cancer*, **13**, 662.

BIELSCHOWSKY, F.—(1958*a*) *Rep. Brit. Emp. Cancer Campgn.* **36**, 456.—(1958*b*) *Proc. int. Symp. on Mammary Cancer*. Ed. L. Severi. Perugia. p. 481.

BONSER, GEORGIANA M.—(1958) *Ibid.*, p. 575.

BOOT, L. M. AND MÜHLBOCK, O.—(1956) *Acta Un. int. Cancr.*, **12**, 569.

CASCHERA, F.—(1960) *Lav. Ist. Anat. Univ. Perugia*, **20**, 17.

COMMITTEE ON STANDARDIZED GENETIC NOMENCLATURE FOR MICE.—(1960) *Cancer Res.*, **20**, 145.

DERINGER, MARGARET K.—(1959) *J. nat. Cancer Inst.*, **22**, 995.

HESTON, W. E.—(1958) *Ann. N.Y. Acad. Sci.*, **71**, 931.

HOWELL, J. S., MARCHANT, J. AND ORR, J. W.—(1954) *Brit. J. Cancer*, **8**, 635.

JULL, J. W.—(1954) *J. Path. Bact.*, **68**, 547.

MARCHANT, JUNE.—(1957) *Brit. J. Cancer*, **11**, 452.—(1959) *Ibid.*, **13**, 652.

MODY, JER.—(1959) Thesis submitted to the University of Leeds for the degree of Ph.D. —(1960) *Brit. J. Cancer*, **14**, 256.

MÜHLBOCK, O. AND BOOT, L. M.—(1960) Symposium on 'Phenomena of the tumor viruses.' New York City.

STREETER, DOROTHY J.—(1960) Thesis submitted to the University of Leeds for the degree of M.Sc.

THE EFFECT OF ADRENALECTOMY ON THE DEVELOPMENT OF TUMOURS INDUCED BY 2-ACETYLAMINOFLUORENE

D. J. PERRY

From the Hugh Adam Department of Cancer Research and the Department of Pathology, Medical School, University of Otago, Dunedin, New Zealand

Received for publication April 8, 1961

THE literature records relatively few studies concerning the role of adrenal deficiency in the development of tumours of the liver and other organs induced by systemically acting compounds. Earlier work by other investigators has been limited to azo dye carcinogenesis and the interpretation of the results obtained have not been uniform (Symeonidis, Mulay and Burgoyne, 1954; Griffin, Richardson, Robertson, O'Neal and Spain, 1955; Eversole, 1957; Da Vanzo and Eversole, 1958; Eversole, 1958; Chany, Aujard and Boy, 1958).

The experiments reported in this paper were undertaken in an attempt to elucidate the role of adrenalectomy in carcinogenesis. 2-Acetylaminofluorene (AAF) was selected as the agent because this compound induces not only liver tumours but also neoplasms of the internal auditory canal, mammary gland, lung and other tissues. With the aid of this agent it seemed possible that one could test whether adrenalectomy would modify the susceptibility of one or more target organs.

MATERIALS AND METHODS

One hundred and fifty male Wistar rats were used in this study. The animals were divided into 5 experimental groups all of which were fed the same diet containing AAF. The animals in Groups I and II were adrenalectomized, those in Groups III, IV and V were not and acted as controls. The rats in Groups II and IV received desoxycorticosterone trimethylacetate (DCT). Group V comprised 7 castrated rats.

The experimental diet had the following composition (per cent by weight): wholemeal flour 70·0, sugar 20·0, casein 8·5, calcium carbonate 1·0, calcium phosphate 0·5. In addition each rat was given 1·5 ml. of cod liver oil and 5 g. of cabbage once a week. Potassium iodide (0·75 mg./l.) was added to the drinking water. The basic diet was weighed out into a feeding bowl and mixed into a moist mash with 0·9 per cent saline. In all cases the amount of fluid used in the preparation of the wet mash was equal to 10 per cent less than the dry weight of the diet. All rats had free access to drinking fluid. Initially this was a solution of 0·9 per cent NaCl in tap water but was later changed to 5 per cent glucose in saline.

The rats in Groups I and II received 10 g. (dry weight) of diet per day, the rats in Groups III, IV and V 12 g. per day. The AAF was incorporated into the diet in amounts sufficient to give each rat the required daily dosage. The carcinogen was added to the diet one week after the animals had been adrenalectomized. Initially the dose of AAF was 4 mg. per day but this was subsequently

lowered to 2 mg. per day because of the high mortality. The AAF was fed for 13–23 weeks, depending upon the daily dosage. All the animals received approximately the same total dosage, i.e. 320 mg. of AAF.

Adrenalectomy was performed under ether anaesthesia using an approach through the paravertebral soft tissues. Great care was taken to avoid rupturing the adrenal capsule, and the glands, together with as much of the surrounding fatty tissue as possible, were removed.

Desoxycorticosterone trimethylacetate was administered by subcutaneous injection. The adrenalectomized animals received 10 mg. every second week and the intact controls 25 mg. every third week. The animals were inspected daily and a careful watch was kept on food consumption. When a group of rats did not eat their daily ration, the amount of food and carcinogen was adjusted. In order to check on the effect of caloric restriction 12 adrenalectomized and 6 intact animals were pair fed.

The rats were weighed weekly at which time a careful examination for tumours was carried out. The rats were housed 6 to a cage. The temperature of the rat room was kept at 72° F. but for added warmth inverted wooden boxes with a small opening cut in one side were placed in the cages containing the adrenalectomized animals.

The rats were killed either when a tumour became palpable or when they appeared likely to die. In addition to representative blocks of tissue from all pathological lesions, at least 4 blocks from the liver were preserved for histological examination. In those adrenalectomized animals where adrenal accessory tissue was not seen at autopsy, the soft tissue from the posterior abdominal wall was taken and serial sections examined for microscopic nodules of adrenal cortical tissue.

RESULTS

Although from the beginning the rats were fed a diet low in potassium and given saline to drink, the mortality was extremely high. This was apparently due to two factors, namely adrenal insufficiency and an increased susceptibility to the toxic effects of AAF. Whereas none of the intact rats died when given 4 mg. of AAF per day, this dose was tolerated only exceptionally by an adrenalectomized animal. It appeared however that death was more often due to lack of adrenal hormones as it was noted that the majority of animals which became ill in the early stages of the experiment presented with a similar clinical picture. They became semicomatose, spastic and cold. This stage was followed by generalized convulsive seizures, deep coma and death. This syndrome suggested a state of hypoglycaemia and a therapeutic trial with intraperitoneal 5 per cent glucose was carried out on a series of 5 moribund animals with highly satisfactory results. However, as the great majority of animals died during the night, controlling the mortality by this means was not possible. That hypoglycaemic attacks were indeed a major factor in the cause of death was confirmed by blood sugar estimations in 5 animals. In one animal the blood glucose was 40 mg. per cent, in all the others it was below 20 mg. per cent. Serum sodium and potassium levels were also estimated in these animals. In all cases the sodium level was within the limits but 2 animals had an elevated serum potassium. Post mortems performed on the animals dying in the first weeks of the experiment showed no gross pathological changes in any of the organs. It therefore appeared that death was due to

a profound disturbance of carbohydrate and possibly potassium metabolism. In all animals coming to post mortem the stomach contained food. This finding, taken in conjunction with the fact that the animals consumed their diet, rules out the possibility that the hypoglycaemia was due to starvation. By adding 5 per cent glucose to the drinking water (0·9 per cent saline) there was some improvement in the survival of the animals but even on this regimen only 16 of the 66 adrenalectomized animals in Group I survived the experimental period of 38–40 weeks. In Group II treated with DCT 10 out of 22 animals survived the required time.

The liver tumours have been classified as either adenomas or carcinomas, the former term has been used to describe those nodules considered to be benign neoplasms. Two main types were observed, solid and cystic.

The solid adenomas were often multiple and measured up to 1·0 cm. in diameter, were white in colour and necrosis was seldom evident macroscopically. Histologically the solid tumours were relatively well differentiated and were often partially encapsulated. The neoplastic cells were arranged in irregular trabeculae and often formed papillary processes or small acini separated by dilated sinusoidal spaces. Occasional tumours were composed of compact sheets of cells in which sinusoidal spaces were inconspicuous.

Small cystic spaces lined by neoplastic epithelial cells were sometimes observed and occasionally in the larger adenomas foci of necrosis were seen. The cells comprising these tumours were either large, and had abundant eosinophilic cytoplasm, or were smaller than normal liver cells and were more basophilic. The cell boundaries were distinct and the nucleus tended to be large and vesicular with prominent nucleoli. An unusual type of adenoma was occasionally observed. This tumour was described by Bielschowsky and Bielschowsky in 1959 and was composed of sheets of large polygonal "plant-like" cells which were full of glycogen.

The small solitary cysts regularly observed in the livers of rats treated with either azo dyes or the aminofluorene derivatives were not considered to be tumours. The term cystic adenoma was restricted to those lesions previously described by Orr (1940) and Opie (1944) as cystadenomas.

Only lesions which were obviously invading the surrounding tissue or had metastasized were classified as carcinoma.

The results obtained are summarized in Table I.

Although all the adrenalectomized rats in Groups I and II received identical treatment, they cannot be regarded as a uniform group. In all but 2 of the rats varying amounts of accessory adrenal tissue was present.

In some cases this tissue was easily identifiable but in others it was only discovered by histological examination of serial sections of the soft tissue from the posterior abdominal wall. For this reason in evaluating the experimental results the adrenalectomized rats in Groups I and II were divided into three sub groups A, B and C, according to the maximum diameter of the adrenal accessory nodule present. In the adrenalectomized rats of Groups I and II no liver tumours were found before the 38th week, whereas in the control rats of Groups III and IV they were present from the 16th week when the earliest palpable liver carcinoma was noted.

In the castrates tumour development was rather slow and the neoplastic lesions were discovered only at autopsy in the 40th weeks. Metastasizing tumours

TABLE I

Group	Adrenalectomy I			Adrenalectomy + DCT II			Controls III	IV	V
								Intact + DCT	Castrate
Subgroup	A	B	C	A	B	C	Intact		
Adrenal accessory	<1·5 mm.	2 mm.	3–4 mm.	<1·5 mm.	2 mm.	3–4 mm.			
Number of animals	7	5	4	8	0	2	34 + 6*	15	7
Duration weeks	38–40	38–40	38–40	25–40	—	38–40	16–40	26–40	16–40	40
Liver wt./100 g. body wt. (mean)	4·1	4·9	5·9	4·4	—	7·5	6·8	6·6	6·5	5·1
Animals with liver tumours	0	3	4	0	—	2	34	6	15	7
Carcinoma	0	1	4	0	—	2	26	5	12	3
Adenoma	0	3	4	0	—	2	34	6	15	7
Carcinoma auditory canal	3	0	0	4	—	1	8	2	5	0
Adenoma lung	0	0	1	0	—	0	1	0	3	0
Carcinoma breast	0	0	1	0	—	0	3	0	3	0
Carcinoma colon	0	0	0	2	—	1	2	0	0	0
Other	0	0	0	0	—	0	0	0	1†	0

* Pair fed to adrenalectomized animals in Group I.

† Leukaemia.

Where both carcinoma and adenoma occur within the same liver both are recorded in the table.

were found in 10 intact controls and 2 adrenalectomized rats which had adrenal accessory tissue of 3–4 mm. in extent.

There was a striking difference in the appearance of the liver in those animals with adrenal tissue meassuring less than 1·5 mm. and those of the controls. The livers of the latter were considerably enlarged and the weight of the organs was often in excess of that which could be attributed to the number and size of the neoplastic lesions present. As well as tumours, areas of necrosis, haemorrhage, scarring and multiple cysts were always present.

In contrast the liver weights of the animals in Groups IA and IIA were all within normal limits and careful inspection failed to reveal any evidence of liver damage, past or present. This finding at autopsy was confirmed by histological examination of numerous blocks of liver tissue. The only abnormality noted was confined to the bile ducts which appeared more prominent and occasionally a few pseudotubules were seen. In 2 livers a microscopic focus of atypical liver cell hyperplasia was seen. The larger the adrenal accessory the more pronounced were the signs of AAF action.

The administration of DCT did not modify the protective effect of adrenalectomy, nor did it have any recognisable effect on the production of tumours in the intact controls.

These results indicate that adrenalectomy protects the livers of rats treated with AAF, the degree of which depends upon the size of the adrenal accessory.

Although the number of extrahepatic tumours occurring in the experimental animals was small, it is obvious that adrenal deficiency did not prevent the development of neoplastic lesions in the external auditory canal and colon.

DISCUSSION

The interpretation of results obtained in long term experiments using the adrenalectomized rat is complicated by the almost constant presence of adrenal cortical tissue. It is well established that if the adrenal capsule is ruptured at the

time of operation remnants of cortical cells may be left behind and these will proliferate and nullify the effect of adrenalectomy. Although it is tempting to ascribe the presence of adrenal cortical tissue in operated animals to imperfections in technique, in this study particular care was taken to remove completely the adrenal and the surrounding fat. In the few cases where the suspicion arose that the adrenal capsule may have been injured the animals were rejected.

MacFarland in 1945 reported that in adrenalectomized rats maintained on saline, adrenal cortical tissue may differentiate from the coelomic mesothelium. Da Vanzo and Eversole (1958) reported that they had found accessory cortical nodules not only in intact rats but also in short term adrenalectomized rats, and concluded that the presence of adrenal accessory tissue did not necessarily imply differentiation from coelomic mesothelium.

In this work post mortem examination of the adrenalectomized animals dying before the 20th week of the experiment showed adrenal accessory tissue in only 2 rats. Had adrenal cortical nodules been present from the beginning it is felt that they would have been discovered by the histological techniques used in this study. It is therefore considered that in some cases at least functional cortical tissue may differentiate from the coelomic mesothelium. Whatever the origin of the adrenal accessory tissue may be, in our experience it apparently needs 20 weeks to grow to a recognizable size. The level of adrenal cortical hormones during the period of administration of the carcinogen must therefore have been very low.

Nevertheless, despite the limitations placed on this type of experiment by the presence of accessory adrenal tissue during the latter part of the experiment the results presented show that although the degree of protection cannot be regarded as absolute, ablation of the adrenals protects the liver against the development of hepatic tumours as long as the adrenal accessory tissue remains below a certain size.

With the exception of Griffin *et al.* (1955) previous investigators, (Symeonidis *et al.*, 1954 ; Eversole, 1957, 1958 ; Chany *et al.*, 1958) working with azo dyes have reported that adrenalectomy inhibits the production of liver tumours. Our results using AAF as the carcinogen are in agreement with their findings. Symeonidis *et al.* observed that desoxycorticosterone acetate prevented tumours of the liver in intact dye fed rats. The results presented in this paper, together with those of Eversole who used 3-methyl-4-dimethylaminoazobenzene (3-Me-DAB) as the carcinogen, makes it unlikely that the mineralocorticoids modify the action of hepatic carcinogens in the presence or absence of the adrenals.

Recently Sidransky, Wagner and Morris (1961) gave a detailed account of histological changes occurring in the livers of rats during the first 4 months of feeding AAF. They showed that the lesions such as mitoses in bile ducts, proliferation of oval cells, nodular hyperplasia and tumour development were less extensive and occurred later in female and castrated male rats than in the intact male.

After adrenalectomy testicular function is depressed and it was therefore considered necessary to include a number of castrates among the intact controls in order to distinguish between the effects due to the lack of androgens and those due to lack of adrenal cortical hormones. As shown in the results, all the castrates had liver tumours by the time the experiment was terminated, although the livers were considerably smaller than in the intact controls. Orchidectomy therefore

delays but does not prevent tumours of the liver and depression of testicular function cannot be the cause of the remarkable insusceptibility of the adrenalectomized rats to AAF.

Caloric restriction can also be ruled out as a major factor responsible for the inhibition of liver tumour development after adrenalectomy. Reduction of food intake to levels consumed by the adrenalectomized animals did not modify the response of the intact animals to AAF.

It has been reported that hypophysectomy (Firminger and Morris, 1955; Skoryna, 1955; Hoffman, 1956; O'Neal and Griffin, 1957; Richardson and O'Neal, 1957; O'Neal, Hoffman, Dodge and Griffin, 1958), as well as thyroidectomy (Bielschowsky and Hall, 1953) protect the liver against aminofluorene derivatives. The question arises whether the effect of ablation of the pituitary and of the thyroid is due to the atrophy of the adrenal which ensues under these conditions. Although atrophy of the adrenals after thyroidectomy is not quite as pronounced as after hypophysectomy, there is no doubt that adrenal function is greatly modified after removal of either gland.

The susceptibility of the liver to the carcinogenic action of the azo dyes can be restored in the hypophysectomized rat by the administration of ACTH (Griffin *et al.*, 1955). However such treatment proved ineffective when diacetylaminofluorene was the carcinogen (O'Neal *et al.*, 1958). This latter finding is difficult to reconcile with the results presented in this paper.

It would seem that lack of adrenal glucocorticoids rather than of mineralocorticoids accounts for the protection seen after ablation of the adrenals since the administration of cortisone restores the susceptibility of the liver to 3-methyl-4-dimethylaminazobenzene (Eversole, 1958) and 2-acetylaminofluorene (unpublished data).

Hypophysectomy (O'Neal *et al.*, 1958) and thyroidectomy (Bielschowsky and Hall, 1953), performed before the administration of the carcinogen not only prevented the development of hepatic tumours, but these procedures also protected the animal from hepatic necrosis and the subsequent cirrhosis. Adrenalectomy has the same effect. These findings raise the question as to whether hepatic injury is an essential factor in the pathogenesis of liver tumours.

Whether ablation of the adrenals inhibits the initiation of neoplastic change in the hepatic parenchymal cells, or whether it prevents growth and progression of already latent neoplastic cells, is a field for further study. It is of interest, however, that in 2 of the adrenalectomized rats with adrenal accessories of less than 1·5 mm. killed 5 months after ceasing 2-AAF feeding, the livers although macroscopically normal, showed an occasional microscopic focus of atypical liver cell hyperplasia. This could be interpreted as evidence for transformation of normal into altered liver cells which however failed to progress to overt tumour formation.

SUMMARY

Adrenalectomy protects the liver of male Wistar rats against the carcinogenic effect of AAF as long as the accessory adrenal tissue does not exceed a critical size. Desoxycorticosterone trimethylacetate did not modify the response of either the adrenalectomized or intact rat to the carcinogen.

I would like to acknowledge my indebtedness to Dr. F. Bielschowsky for his unfailing encouragement and advice and also to Dr. Marianne Bielschowsky for her very great assistance in caring for the experimental animals.

REFERENCES

BIELSCHOWSKY, F. AND BIELSCHOWSKY, M.—(1959) In Ciba Foundation Symposium on Carcinogenesis. London (Churchill), p. 95.

Idem AND HALL, W. H.—(1953) *Brit. J. Cancer*, **7**, 358.

CHANY, E., AUJARD, C. AND BOY, J.—(1958) *C.R. Soc. Biol., Paris*, **152**, 275.

DA VANZO, J. P. AND EVERSOLE, W. J.—(1958) *Cancer Res.*, **18**, 796.

EVERSOLE, W. J.—(1957) *Proc. Soc. exp. Biol., N.Y.*, **96**, 643.—(1958) *Lav. Ist. Anat. Univ. Perugia*, **18**, 25.

FIRMINGER, H. I. AND MORRIS, H. P.—(1955) *Fed. Proc.*, **14**, 402.

GRIFFIN, A. C., RICHARDSON, L. H., ROBERTSON, C. H., O'NEAL, A. M. AND SPAIN, J. D.—(1955) *J. nat. Cancer Inst.*, **15**, 1623.

HOFFMAN, H. E.—(1956) Thesis. *Univ. Microfilm Publ.* No. 16, p. 847. Stanford University.

MACFARLAND, W. E.—(1945) *Anat. Rec.*, **93**, 233.

O'NEAL, M. A. AND GRIFFIN, A. C.—(1957) *Proc. Amer. Ass. Cancer Res.*, **2**, 236.

Idem, HOFFMAN, H. E., DODGE, B. G. AND GRIFFIN, A. C.—(1958) *J. nat. Cancer Inst.*, **21**, 1161.

OPIE, E. L.—(1944) *J. exp. Med.*, **80**, 231.

ORR, J. W.—(1940) *J. Path. Bact.*, **50**, 393.

RICHARDSON, H. L. AND O'NEAL, M. A.—(1957) *Proc. Amer. Ass. Cancer Res.*, **2**, 242.

SIDRANSKY, H., WAGNER, B. P. AND MORRIS, H. P.—(1961) *J. nat. Cancer Inst.*, **26**, 151.

SKORYNA, S. C.—(1955) *Proc. Canad. Cancer Res.*, Conf. **1**, 107.

SYMEONIDIS, A., MULAY, A. S. AND BURGOYNE, F. H.—(1954) *J. nat. Cancer Inst.*, **14**, 805.

RATIO DEPENDENT SYNERGISM IN AZO-DYE CARCINOGENESIS*

J. C. ARCOS† AND G. W. GRIFFITH

From the Cancer Research Laboratory, J. Hillis Miller Health Center University of Florida, Gainesville, Florida

Received for publication April 8, 1961

A SCARCITY of information exists in the literature on the hepatocarcinogenic effects of combinations of azo dyes, similar to the competition in epithelial carcinogenesis between polycyclic hydrocarbons as described first by Lacassagne, Buu-Hoi and Rudali (1945). In the reports available on synergism among azo carcinogens only the synergistic effect of 4-dimethylaminoazobenzene (DAB) and 4′-methyl-DAB has been confirmed (MacDonald *et al.*, 1952; Corre-Hurst *et al.*, 1953). The final assessment of the synergism between DAB and 3′-methyl-DAB (3′-Me-DAB) described in the 1953 report will require further experiments, however, in view of the fact that the authors found a higher tumor incidence with DAB than with 3′-Me-DAB, which is the inverse of the generally accepted relative carcinogenicites of the two compounds. Inhibitory effect among azo carcinogens has been reported by Crabtree (1955) who described inhibition of DAB induced hepatic tumorigenesis by certain non-carcinogenic derivatives of azobenzene.

These observations have now been extended by studying the carcinogenic effect of dietary mixtures of the potent hepatic carcinogen, 3′-Me-DAB, of its relatively inactive isomer, 2-Me-DAB, and of the parent compound DAB. This report describes the synergistic effect of 3′-Me-DAB and of 2-Me-DAB when these dyes are present in a 1 : 2 proportion in the diet. However, when these two azo compounds are present in a ratio of 1 : 1, the synergistic effect is abolished, and the comparison of the tumor incidences may be considered suggestive of a limited inhibitory effect of 2-Me-DAB on 3′-Me-DAB tumorigenesis. In relation to this biological effect the phase diagram of 2-Me and 3′-Me-DAB has been studied to bring evidence for their actual similarity of spatial configuration.

MATERIALS AND METHODS

Care and feeding of animals.—Sprague-Dawley male rats, weighing 180 to 230 g. at the beginning of the experiment and housed two to a cage, were used. The rats were fed a semi-synthetic diet, called hereafter basal diet, similar to the diet No. 2 used by Miller, Miller and Finger (1957). In the present diet, however, the previous salt mixture (Miller, MacDonald and Miller, 1955) has been substituted by the commercially available Salt Mixture W (Nutritional Biochemicals Corporation, Cleveland 28, Ohio). In addition to this, the diet was now supplemented

* Presented at the Fifty-first Annual Meeting of the American Association for Cancer Research, Inc., Chicago, Illinois, April 8–10, 1960 (*Proc. Am. Assoc. Cancer Res.*, **3**, 92, 1960).

† Present address: U.S. Public Health Service Hospital, Research Laboratory, 210 State Street and Departments of Medicine and Biochemistry, Tulane University Medical School, New Orleans, Louisiana.

with 1·6 mg./kg. of Menadione (2-methyl-1,4-naphthoquinone) and also with 14 mg./kg. of zinc sulfate heptahydrate since the Salt mixture W does not contain zinc, which is essential to the development of the rat (Farris and Griffith, 1949). The azo compounds, DAB, 3′-Me-DAB and 2-Me-DAB were incorporated in the basal diet at the levels stated in Table I. The rats were fed basal diet for at least

TABLE I.—*Hepatic Tumour Incidence with Combinations of 3′-Me-DAB, 2-Me-DAB and DAB*

Group	Diet	Number of rats started	Incidence of liver tumors* (months of continuous feeding)					
			3½	4	4½	6	7	8
A	0·02 per cent 3′-Me-DAB + 0·04 per cent 2-Me-DAB	23	—	—	0/20	—	5/20†	—
B	0·02 per cent 3′-Me-DAB	23	—	—	0/23	—	1/23	—
C	0·04 per cent 2-Me-DAB	23	—	—	0/23	—	0/22	—
D	0·035 per cent 3′-Me-DAB + 0·035 per cent 2-Me-DAB	24	1/24‡	—	10/24	21/23	—	—
E	0·035 per cent 3′-Me-DAB	24	0/24	—	14/24	24/24	—	—
F	0·06 per cent 2-Me-DAB 3 mo. → 0·054 per cent DAB	20	—	1/18	—	—	—	4/17
G	Basal diet 3 mo. → 0·054 per cent DAB	20	—	0/20	—	—	—	3/18

* Number of rats with tumors/number of rats alive.
† In addition to these five tumor bearing animals, a sixth had a teratoma of a kidney.
‡ Hepatic tumors failed to develop in an additional group of 19 rats maintained on this diet for 3½ months. This would bring the overall tumor incidence to 1/43.

1 week prior to the feeding of the dye containing diet. All diets and water were *ad libitum*. For the intermittent examinations for liver tumors exploratory laparotomy was performed under ether anesthesia. At the end of the respective experiments all survivors were sacrificed for final tumor count and extensive autopsy. All macroscopically visible nodules found during exploratory laparotomy or at the termination of experiments were examined histopathologically. When no gross tumor was apparent and no small tumor foci were visible, the liver was described as "normal" regardless of the well known cellular changes preceding the emergence of tumors, which was the criterion used by Crabtree (1955).

Preparation of compounds and determination of phase diagrams.—DAB was obtained commercially. 3′-me DAB and 2-Me-DAB were prepared in our laboratory following the standardized procedure of Miller, Sapp and Miller (1949). 4-Hydroxyazobenzene was obtained in a similar way by coupling benzenediazonium-chloride to phenol in 70 per cent aqueous ethanol in the presence of 1·5 mole NaOH for each mole of phenol. At the end of coupling, the reaction mixture was brought to pH 7 and the precipitation of the dye enhanced by adding a saturated solution of sodium acetate.

Once crystallized samples were used for feeding, and three times crystallized and carefully dried samples were used for the determination of the phase diagrams. Initial thawing points and final melting points were taken in triplicate with a capillary arrangement in a Thiele tube fitted with a standardized thermometer and a magnifying lens. All melting points are corrected. The variations of the final melting points were within $\pm 0{\cdot}5$ per cent.

RESULTS AND DISCUSSION

Animal experiments

The tumor incidences given for Groups A, B and C of Table I show marked synergism when 0·04 per cent 2-Me-DAB and 0·02 per cent 3′-Me-DAB are present in mixture in the diet fed for 7 months. That the observed effect corresponds to true synergism is indicated by the fact that in Group A the two compounds together (at the concentrations as they are present singly in Groups B and C) produced 5 tumor bearers in 20 rats (25 per cent incidence), compared with 1 tumor bearer produced by these dyes administered separately to a total of 45 rats in Groups B and C (2·2 per cent). This difference is statistically significant, since $P < 0{\cdot}01$ using Fisher's exact test for 2×2 contingency tables. On the other hand, a comparison of Groups C, D and E indicates that when these two dyes are present in the diet in equal proportion, no synergism is observed although the level of 2-Me-DAB in the test Group D (0·035 per cent) is similar to that in test Group A (0·04 per cent). For the difference between the tumor incidences in Groups C plus E, and Group D, at $4\frac{1}{2}$ months $\chi^2 = 0{\cdot}545$ was found, corresponding to $P \simeq 0{\cdot}45$ on 1 degree of freedom.

Two alternative assumptions may be made for the absence of synergism in the latter experiment: (1) If the tumor incidence values in these various groups are plotted against the respective time intervals, it may be seen that the slope of the curve of Group E is much greater than the one of the synergistic Group A. For this reason the absence of synergism in Group D may be interpreted as resulting from a "covering" of the synergistic effect of 0·035 per cent 2-Me-DAB by the rapidity of tumour development due to the presence of 3′-Me-DAB at the 0·035 per cent level. (2) When both 2-Me and 3′-Me-DAB are fed at identical levels of 0·035 per cent each (Group D), competitive antagonism may occur at certain cellular binding sites compensating the synergistic effect of 2-Me-DAB. This possibility of competitive antagonism is also suggested by: (*a*) the presence of both compounds in all liver cell fractions of treated rats (Price *et al.*, 1949, 1950; Hultin, 1956); (*b*) the close similarity of the spatial configurations of the two dyes (see "Phase Diagrams"), and (*c*) the fact that both 2-Me and 3′-Me-DAB are linked in the soluble cytoplasm to the same electrophoretic protein fraction (Sorof *et al.*, 1951; Wirtz and Arcos, 1958). The comparison of the tumor incidences at $4\frac{1}{2}$ months in Group D (10/24) and Group E (14/24) may actually be considered as suggestive of a limited inhibition of 3′-Me-DAB tumorigenesis by 2-Me-DAB. The statistical significance of this apparent difference is, however, low ($P \simeq 0{\cdot}30$). Unfortunately, due to the difficulties in carrying out large numbers of laparotomies within short intervals of time and because these experiments had to be terminated, a more solid assessment of the latter data could not be obtained.

In another experiment, feeding of 2-Me-DAB preceeded feeding the medium active DAB (Groups F and G). The rats of the test Group F were fed 0·06 per cent 2-Me-DAB for 3 months, which has been shown (Miller and Miller, 1947) to be the time when maximum level of bound dye is reached with this compound under similar dietary conditions. After this period, feeding was immediately continued with 0·054 per cent DAB. The control Group G was fed basal diet for 3 months and then fed the same level of DAB. No difference could be observed between the tumor incidences in these groups ($P > 0{\cdot}90$). Thus, previous saturation of cellular binding sites with 2-Me-DAB does not affect DAB induced tumorigenesis.

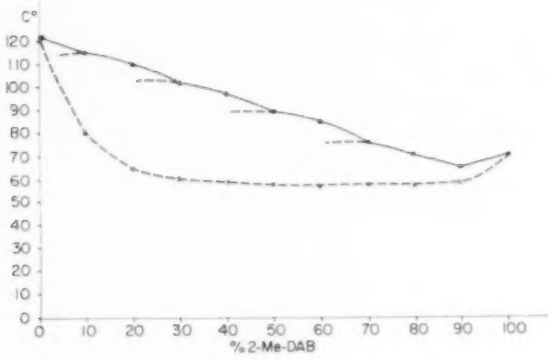


FIG. 1.

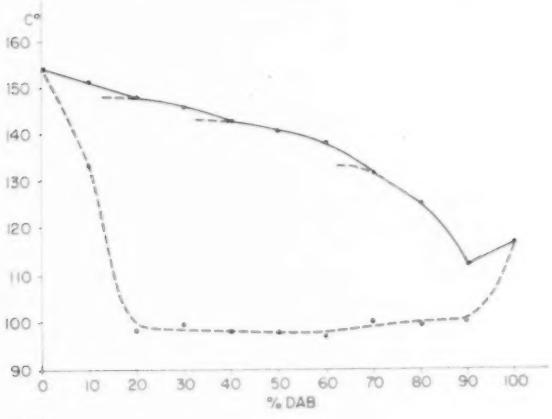


FIG. 2.

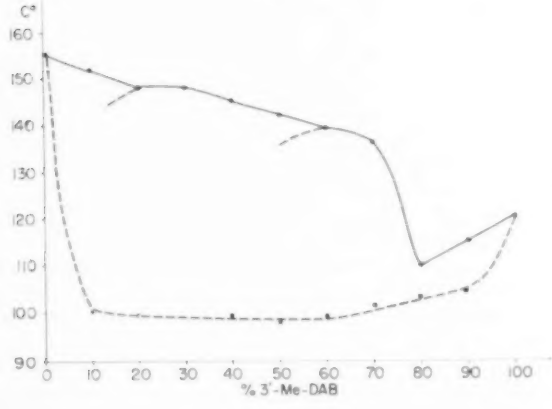


FIG. 3.

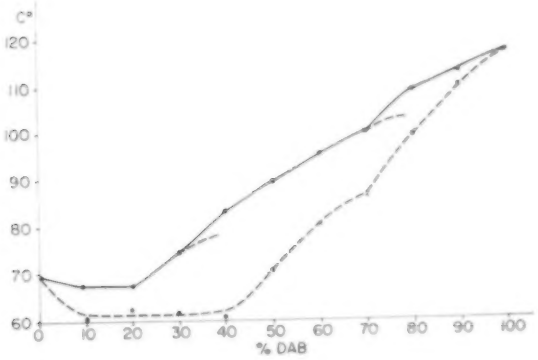


FIG. 4.

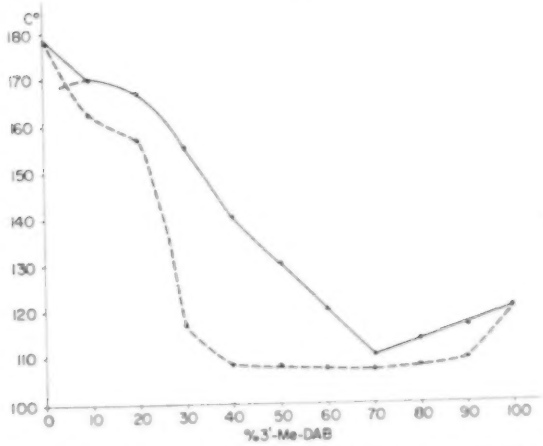


FIG. 5.

FIG. 1 to 5.—Phase diagrams of binary mixtures of azo dyes showing polymorphic transformations and regions of continuous miscibility. Final melting points (solid line) varied within ±0·5 per cent. The dotted line curves give the initial thawing points. The figures correspond to the following pairs of compounds: Fig. 1, 3′-Me-DAB plus 2-Me-DAB; Fig. 2, DAB plus 4-hydroxyazobenzene; Fig. 3, 3′-Me-DAB plus 4-hydroxyazobenzene; Fig. 4, DAB plus 2-Me-DAB; Fig. 5, 3′-Me-DAB plus 20-methylcholanthrene.

Histologically the tumors were classified mainly as hepatomas and cholangiocarcinomas and these two histopathological types were observed in about equal proportion. Three of the 5 tumors found in Group A fed 0·04 per cent 2-Me-DAB plus 0·02 per cent 3′-Me-DAB were, however, cholangiomas.

The cellular mechanism that may account for the observed synergistic effect must involve more than one receptor site (*cf.* Ariens, van Rossum and Simonis, 1956; Veldstra, 1956) and this is consistent with the general distribution of bound aminoazo dyes among all cell components of the liver (Price *et al.*, 1949, 1950; Hultin, 1956). The liver enzyme systems which metabolize various drugs and inactivate carcinogenic azo dyes by *N*-demethylation, azo double bond reduction

and ring hydroxylation (*e.g.*, Mueller and Miller, 1949; Conney *et al.*, 1957; Conney, Miller and Miller, 1957; Conney *et al.*, 1959) have been identified in the "microsome" fraction. These enzyme systems are altered and functionally eliminated at high levels of feeding 3′-Me-DAB (Miller, Miller and Brown, 1952) at a rate which may be expected to be linear with the concentration of the dye fed. An approximate proportionality is in fact shown by the carcinogenicities of 0·06 per cent and 0·035 per cent 3′-Me-DAB diets: 100 per cent tumour incidence has been observed in a group of 16 rats after 4 months of continuous feeding 0·06 per cent 3′-Me-DAB, while Table I indicates that with 0·035 per cent diet only 14 tumour bearing rats out of 24 have been found after $4\frac{1}{2}$ months (Group E). This approximate linearity of the dose-response fails, however, when the responses to the 0·035 and 0·02 per cent diets are compared, since no tumor bearing animal is found when the latter diet is fed for $4\frac{1}{2}$ months to 23 rats (Group B). The relative inactivity of the 0·02 per cent 3′-Me-DAB diet may be due to a rate of metabolic inactivation which balances the rate of arrival of the dye in the liver. Consequently, from the standpoint of the process of carcinogenesis, the microsomal enzyme systems inactivating 3′-Me-DAB may be considered "sites of loss" in the sense described by Veldstra (1956).

Carcinogenesis may result then from: (*a*) intake of 3′-Me-DAB in excess over the metabolic capacity of the enzyme systems in the microsomes, which excess will be available for alteration of this cell organelle proper (Arcos and Arcos, 1958; Porter and Bruni, 1959), and of other cellular components, *e.g.* the mitochondria (Arcos, Griffith and Cunningham, 1960), at a rate which is proportional to the amount of non-metabolized dye, such as may be the case with the 0·035 and 0·06 per cent diets, or (*b*) competition of 2-Me and 3′-Me-DAB at "sites of loss" (*cf.* Veldstra, 1956) making 3′-Me-DAB available at other cellular sites the alterations of which are involved in the succession of steps leading to carcinogenesis (Arcos, Gosch and Zickafoose, 1961).

Phase diagrams

Synergism and antagonism as the response to simultaneously present biologically active compounds often involves close steric molecular similarity (*e.g.* Veldstra, 1956). Because of steric hindrance between the 2-methyl group and the α-azo nitrogen in 2-Me-DAB (Arcos and Arcos, 1958), it may be expected that the spatial configuration of this compound and of 3′-Me-DAB may not be so closely similar as suggested by the simple observation of the structural formulae. Steric hindrance in 2-Me-DAB is also suggested by the high basicity of the amino nitrogen (Cilento, 1960), because of interference with the resonance involving the azo double bond and consequent increase of the electron density at the amino nitrogen. For these reasons, the phase diagram of these dyes, and comparatively of binary mixtures of other azo compounds have been studied since it is well known that polymorphic transformations or continuous mixed crystal formation, that may be detected in such diagrams, indicate interchangeability in mixed crystal lattices (*e.g.* Eitel, 1945) which in turn is dependent on close steric similarity or complementarity (*e.g.* Timmermans, 1939; Fredga, 1945).

The phase diagrams obtained with mixtures of various azo dyes suggest that the interchangeability of 3′-Me-DAB and 2-Me-DAB in their mixed crystal lattices is relatively higher than in mixed crystals of certain structurally related azo dyes. In fact, the phase diagram of these two compounds (Fig. 1) indicates the existence

of at least four regions of polymorphic transformations, in addition to a rather flat eutectic region around 90 per cent 2-Me-DAB. Moreover, the flatness of this region and the even concavity of the thawing point curve (dotted line) relatively to the final melting point curve (solid line) is suggestive, that other polymorphic transformations might have remained undetected and so that the two compounds possibly form a nearly continuous series of solid solutions. The temperature differences involved in the regions of polymorphic transformations are significant because of the narrow range of the final melting points ($\pm$0·5 per cent). Thus, there is no overlapping between the minima representing these polymorphic transformations and the points (with the respective $\pm$0·5 per cent ranges) corresponding to them on a curve which would join the remaining points of the final melting point curves. Decreasing numbers of polymorphic transformations may be observed in the phase diagrams of other azo dyes (Fig. 2 to 5). However, the curve of the thawing points in Fig. 4 is suggestive that, also with DAB plus 2-Me-DAB, other regions of polymorphic transformations may exist between 40 and 100 per cent DAB. The anomaly in Fig. 2 and 3 that sharp eutectic points coexist with rather flat shoulders up to 60–70 per cent DAB or 3′-Me-DAB, may be due to more stable molecular combinations by interaction between the phenolic hydroxyl in 4-hydroxyazobenzene and the basic nitrogen in the two aminoazo dyes. In the phase diagram of 3′-Me-DAB plus 20-methylcholanthrene (Fig. 5) a very noticeable polymorphic transformation has been observed at 10 per cent 3′-Me-DAB, in addition to an expected sharp eutectic point.

The above phase diagrams recall the interesting work of Müller, Görlich and Kahofer (1954) who observed a correlation between mixed crystal formation in binary mixtures of polycyclic hydrocarbons and antagonism of these compounds in epithelial carcinogenesis. Applying the well known concept that for heterogenous organic catalysis a correlation of molecular geometry must exist between the catalytic site and the organic substrate, these authors suggested that the mixed crystal formation of polycyclic hydrocarbons may imply interchangeability at cellular receptor sites.

SUMMARY

1. There is synergism in hepatic tumor induction when a diet containing 0·04 per cent 2-Me-DAB plus 0·02 per cent 3′-Me-DAB is fed to rats. This synergism is abolished by raising the concentration of 3′-Me-DAB to 0·035 per cent. Prolonged previous feeding with 0·06 per cent 2-Me-DAB does not affect the tumor incidence produced by a 0·054 per cent DAB diet.

2. Regions of polymorphic transformations that may be observed in the phase diagram of 2-Me-DAB plus 3′-Me-DAB suggest that, in spite of the steric hindrance present in the former compound, there is a close similarity in their spatial configurations. For comparison, phase diagrams of pairs of other azo dyes have been determined.

The authors are greatly indebted to Dr. Steven P. H. Mandel for the statistical calculations and related evaluation of the results, to Dr. Mary F. Argus for discussion and criticism of the manuscript, to Dr. Cornelia Hoch-Ligeti and Dr. Joseph Simon for the histopathological examinations, and to Dr. Francis E. Ray for support during the early stages of these investigations.

This work was supported in part by the U.S. Public Health Service Research Grant C–4351.

REFERENCES

ARCOS, J. C. AND ARCOS, M.—(1958) *Biochim. biophys. Acta*, **28**, 9.—(1958) *Arzneimittel-forschung*, **8**, 486.

Idem, GOSCH, H. H. AND ZICKAFOOSE, D.—(1961) *J. biophys. biochem. Cytol.*, in press.

Idem, Griffith, G. W., and Cunningham, R. W.—(1960) *Ibid.*, **7**, 49.

ARIENS, E. J., VAN ROSSUM, J. M. AND SIMONIS, A. M.—(1956) *Arzneimittelforschung*, **6**, 737.

CILENTO, G.—(1960) *Cancer Res.*, **20**, 120.

CONNEY, A. H., BROWN, R. R., MILLER, J. A. AND MILLER, E. C.—(1957) *Ibid.*, **17**, 628.

Idem, GILLETTE, J. R., INSCOE, J. K., TRAMS, E. R. AND POSNER, H. S.—(1959) *Science*, **130**, 1478.

Idem, MILLER, E. C. AND MILLER, J. A.—(1957) *J. biol. Chem.*, **228**, 753.

CORRE-HURST, L., BUU-HOI, N. P., ROYER, R., AND BIZZINI, B.—(1953) *Bull. Ass. franç. Cancer*, **40**, 397.

CRABTREE, H. G.—(1955) *Brit. J. Cancer*, **11**, 310.

EITEL, H.—(1945) "Die Heterogenen Schmelzgleichgewichte Silikatischer Mehrstoff-Systeme." Leipzig (Barth), pp. 9–12.

FARRIS, E. J. AND GRIFFITH, J. Q.—(1949) 'The Rat in Laboratory Investigation.' Philadelphia (Lippincott), pp. 80, 98.

FREDGA, A.—(1945) 'On the Use of Melting Point Curves for the Establishment of Steric Relationships between Optically Active Compounds,' in Tiselius and Pedersen, Ed., 'The Svedberg 1884–1944.' Uppsala (Almqvist & Wiksells), pp. 261–273.

HULTIN, T.—(1956) *Exp. Cell Res.*, **10**, 71, 697.

LACASSAGNE, A., BUU-HOI, N. P. AND RUDALI, G.—(1945) *Brit. J. exp. Path.*, **26**, 5.

MACDONALD, J. C., MILLER, E. C., MILLER, J. A. AND RUSCH, H. P.—(1952) *Cancer Res.*, **12**, 50.

MILLER, E. C., MACDONALD, J. C. AND MILLER, J. A.—(1955) *Ibid.*, **15**, 320.

Idem and Miller, J. A.—(1947) *Ibid.*, **7**, 468.

Idem, MILLER, J. A. AND BROWN, R. R.—(1952) *Ibid.*, **12**, 282.

MILLER, J. A., MILLER, E. C. AND FINGER, G. C.—(1957) *Ibid.*, **17**, 387.

Idem, SAPP, R. W. AND MILLER, E. C.—(1949) *Ibid.*, **9**, 652.

MÜLLER, A., GÖRLICH, P. AND KAHOFER, L.—(1954) *Mh. Chem.*, **85**, 906.

MUELLER, G. C. AND MILLER, J. A.—(1949) *J. biol. Chem.*, **180**, 1125.

PORTER, K. R. AND BRUNI, C.—(1959) *Cancer Res.*, **19**, 997.

PRICE, J. M., MILLER, E. C., MILLER, J. A. AND WEBER, G. M.—(1949) *Ibid.*, **9**, 398.—(1950) *Ibid.*, **10**, 18.

SOROF, S., COHEN, P. P., MILLER, E. C. AND MILLER, J. A.—(1951) *Ibid.*, **11**, 383.

TIMMERMANS, J.—(1939 *Bull. Soc. chim. Belges*, **48**, 33.

VELDSTRA, H.—(1956) *Pharmacol. Rev.*, **8**, 339.

WIRTZ, G. H. AND ARCOS, J. C.—(1958) *Experientia*, **14**, 177.

THE BEHAVIOUR *IN VITRO* OF LIVER FROM RATS TREATED WITH A CARCINOGEN

J. O. LAWS AND SALLIE YATES

From the Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds, 2

Received for publication January 27, 1961

CHANGES in the liver of rats fed the carcinogen 2-acetylamino-fluorene for a period have been described by a number of workers (Skoryna and Webster, 1951; Laws, Mabille, Royer and Rudali, 1952). Briefly there is some initial damage which may amount to visible necrosis, followed by nodular hyperplasia which becomes more marked as the feeding of the carcinogen continues. This is accompanied by cyst formation and finally hepatomas arise often multicentrically. In a recent paper one of the authors (Laws, 1959) showed that if partial hepatectomy is carried out on rats which have received the carcinogen for a period too short to produce obvious change, regeneration of the liver nevertheless is delayed. When it does occur, after a period of five to fourteen days, it is abnormal in type. The present paper describes the behaviour of such abnormally regenerating livers when explanted *in vitro*. The conditions of culture have been chosen to maintain the cells in a healthy condition for a period without stimulation so that their behaviour will reflect their condition in the organ prior to that sacrifice of the animal.

MATERIALS AND METHODS

Animals.—All the experiments were carried out on 3 to 4 months old male rats of the Leeds strain. These animals are of Wistar stock and are characterised by a slow response to the carcinogen, the first overt histological changes in intact animals not occurring until the seventh to ninth weeks, in contrast to a number of other strains in which they are found as early as the third week.

Carcinogen.—The carcinogen 2-acetylaminofluorene (L. Light and Co.) was incorporated in a meal diet at a strength of 0·1 per cent. This diet supplied by the North Eastern Agricultural Co-operative Society, Aberdeen satisfies all nutritional requirements. (For further details of the preparation see Laws, 1959).

Partial hepatectomy.—This operation, by the method of Higgins and Anderson (1931) removes approximately two-thirds of the liver substance.

Tissue culture.—Explants consisting of small portions of the liver were prepared by cutting the organ with a sharp scalpel in balanced salt solution (Earle's). They were then washed in changes of the medium to be used. In pilot experiments these were embedded in plasma clot, or in collagen gel (Ehrmann and Gey, 1956), but these substrates were not found to be satisfactory. Plasma clot tended to inhibit epithelial migration until the clot had been dissolved, after which the explants tended to separate from the surface. Collagen proved difficult to manage

and showed no advantage over placing the explants directly on the glass surface. In the experiments reported therefore explants were placed directly on the surface of roller tubes, allowed to adhere only for the time needed to deal with a series of fifteen tubes, then medium was added and the tubes were stoppered. They were immediately placed in the roller drum, which turned at a speed of 30 revolutions per hour and kept there for the duration of the experiment.

Medium.—The medium used in these experiments was designed to allow cell migration without stimulation of growth, and to maintain the cells in health for a period of about ten days, since interest was concentrated in the initial behaviour of the explant. Its constitution was as follows:—

	per cent.
Calf Serum, (Oxoid)	40
Synthetic medium 199 (Parker)	40
Earle's balanced salt solution	20

Explantation.—For control normal cultures explants were taken from three weeks old rats and from adult rats both before and at intervals after partial hepatectomy. In the carcinogen-treated group partial hepatectomy was performed three weeks after the start of feeding with acetylaminofluorene and administration of the carcinogen was continued after the operation until the animals were killed for culture.

Staining.—Cultures were observed under the microscope daily and representative examples were fixed at intervals in formol saline or methyl alcohol. Some were stained by the Periodic-acid Schiff (PAS) method and some by Giemsa's stain. The normal histological procedures were used in each case. PAS stained specimens were counterstained with Harris's haematoxylin. In examining fresh specimens of liver the aniline blue staining technique of one of the authors (Laws, 1961) was also used.

TABLE I.—*Migration from Explants of Normal Liver*

	Days from explantation	Explants showing macrophages and fibroblasts only (per cent.)	Explants showing epithelial cells (per cent.)
Three weeks old rats	2	65	5
	6		85 Total
	7		95 400 explants
Normal adult rats	2	45	
	6	5	55 Total
	7	5	55 550 explants
Adult rats 24 hours after hepatectomy	2	51	
	6	16	83 Total
	7	16	83 180 explants
Adult rats three days after hepatectomy	2	45	
	6	55	90 Total
	7	55	90 180 explants
Adult rats seven days after hepatectomy	Active migration was seen from only a few explants		

(Sixty explants are made from each animal sacrificed.)

TABLE II.—*Migration from Explants of Carcinogen-treated Liver*

All these animals received carcinogen for three weeks and were then partially hepatectomised.

	Days from explantation	Explants showing macrophages and fibroblasts only (per cent)	Explants showing epithelial cells (per cent)	
Three days after hepatectomy	2	20	—	
	5	—	20	Total
	10	—	20	180 explants
Five days after hepatectomy	1	40	—	
	4	22	23	Total
	7	—	45	60 explants
Seven days after hepatectomy	3	50	5	
	7	—	55	Total
	10	—	55	300 explants
Ten days after hepatectomy	2	52	3	
	7	—	55	Total
	10	—	55	300 explants
Fourteen days after hepatectomy	2	4	39	
	7	—	43	Total
	10	—	43	240 explants

(Sixty explants are made from each animal sacrificed.)

RESULTS

The quantitative results, that is the number of explants from the various groups of animals which showed active migration of cells are shown in Tables I and II. It will be seen that in most cases a migration of macrophage and fibroblast cells preceded that of epithelial cells. In fact no explants of normal liver showed epithelial cell migration without preceding activity of mesenchymal cells. In the carcinogen group explanted fourteen days after hepatectomy the epithelial activity was precocious, in some cases no macrophage or fibroblast cells were seen. The livers of normal young adult rats show active epithelial migration, but this is reduced following hepatectomy. In the carcinogen-treated animals active migration occurs after operation but the figures can only be understood fully in reference to the histological picture of these livers and the types of migration seen, which is described below.

In the normal livers of all types the general picture of migration is similar. Following the emergence of a variable number of small macrophage cells, and of fibroblasts which may form a loose network, sheets of epithelial cells begin to grow out from a number of sites at the edge of the explant. This sheet gradually extends for a variable distance, maintaining a general close adhesion of cells which are generally similar in size and appearance. In the three-week old rats this sheet may extend for 1–2 mm. from the edge of the explant, and some cells may break away in small clusters but in adult animals it seldom reaches more than 0·5 mm. The cells are large, 70–100μ in diameter, and the nuclei show a large nucleolus and resemble those seen in normal liver. Binucleate cells are quite common. When stained by the PAS method the cells may at first show little

and showed no advantage over placing the explants directly on the glass surface. In the experiments reported therefore explants were placed directly on the surface of roller tubes, allowed to adhere only for the time needed to deal with a series of fifteen tubes, then medium was added and the tubes were stoppered. They were immediately placed in the roller drum which turned at a speed of 10 revolutions per hour and kept there for the duration of the experiment.

Medium.—The medium used in these experiments was designed to allow cell migration without stimulation of growth, and to maintain the cells in health for a period of about ten days, since interest was concentrated in the initial behaviour of the explant. Its constitution was as follows :—

	per cent
Calf Serum (Oxoid) . . .	40
Synthetic medium " 199 " (Parker) .	40
Earle's balanced salt solution . .	20

Explantation.—For control normal cultures explants were taken from three-week old rats, and from adult rats both before and at intervals after partial hepatectomy. In the carcinogen-treated group partial hepatectomy was performed three weeks after the start of feeding with acetylaminofluorene and administration of the carcinogen was continued after the operation until the animals were killed for culture.

Staining.—Cultures were observed under the microscope daily and representative examples were fixed at intervals in formol saline or methyl alcohol. Some were stained by the Periodic-acid Schiff (PAS) method and some by Giemsa's stain. The normal histological procedures were used in each case. PAS stained specimens were counterstained with Harris's haematoxylin. In examining fresh specimens of liver the aniline blue staining technique of one of the authors (Laws, 1961) was also used.

TABLE I.—*Migration from Explants of Normal Liver*

	Days from explantation	Explants showing macrophages and fibroblasts only (per cent)	Explants showing epithelial cells (per cent)	
Three weeks old rats	2	62	3	
	4	—	65	Total
	7	—	65	600 explants
Normal adult rats	2	42	—	
	4	5	51	Total
	7	5	51	350 explants
Adult rats 24 hours after hepatectomy .	2	51	—	
	4	16	35	Total
	7	16	35	180 explants
Adult rats three days after hepatectomy .	2	45	—	
	4	25	20	Total
	7	25	20	180 explants
Adult rats seven days after hepatectomy .	Active migration was seen from only a few explants			

(Sixty explants are made from each animal sacrificed.)

TABLE II.—*Migration from Explants of Carcinogen-treated Liver*

All these animals received carcinogen for three weeks and were then partially hepatectomised.

	Days from explantation	Explants showing macrophages and fibroblasts only (per cent)	Explants showing epithelial cells (per cent)	
Three days after hepatectomy	2	20	—	
	5	—	20	Total
	10	—	20	180 explants
Five days after hepatectomy	1	40	—	
	4	22	23	Total
	7	—	45	60 explants
Seven days after hepatectomy	3	50	5	
	7	—	55	Total
	10	—	55	300 explants
Ten days after hepatectomy	2	52	3	
	7	—	55	Total
	10	—	55	300 explants
Fourteen days after hepatectomy	2	4	39	
	7	—	43	Total
	10	—	43	240 explants

(Sixty explants are made from each animal sacrificed.)

RESULTS

The quantitative results, that is the number of explants from the various groups of animals which showed active migration of cells are shown in Tables I and II. It will be seen that in most cases a migration of macrophage and fibroblast cells preceded that of epithelial cells. In fact no explants of normal liver showed epithelial cell migration without preceding activity of mesenchymal cells. In the carcinogen group explanted fourteen days after hepatectomy the epithelial activity was precocious, in some cases no macrophage or fibroblast cells were seen. The livers of normal young adult rats show active epithelial migration, but this is reduced following hepatectomy. In the carcinogen-treated animals active migration occurs after operation but the figures can only be understood fully in reference to the histological picture of these livers and the types of migration seen, which is described below.

In the normal livers of all types the general picture of migration is similar. Following the emergence of a variable number of small macrophage cells, and of fibroblasts which may form a loose network, sheets of epithelial cells begin to grow out from a number of sites at the edge of the explant. This sheet gradually extends for a variable distance, maintaining a general close adhesion of cells which are generally similar in size and appearance. In the three-week old rats this sheet may extend for 1–2 mm. from the edge of the explant, and some cells may break away in small clusters but in adult animals it seldom reaches more than 0·5 mm. The cells are large, 70–100μ in diameter, and the nuclei show a large nucleolus and resemble those seen in normal liver. Binucleate cells are quite common. When stained by the PAS method the cells may at first show little

staining but in older sheets many of the cells will contain red-staining glycogen granules, often in large quantities. Under the conditions used the cells tended to disintegrate between seven and ten days after explantation and the cultures were then discarded.

In the carcinogen treated livers the behaviour of the explants varied with the period which had elapsed between hepatectomy and explantation. To understand the results it is first necessary to consider the histology of such livers. This is reported in detail in an earlier paper (Laws, 1959) but briefly is as follows: The liver in the unoperated carcinogen treated rat shows little obvious histological change but explants from such livers show little outgrowth. When it occurs it consists of sheets of loosely adherent parenchymal cells of small extent. Following hepatectomy there are two phases. During the first phase the continued presence of the carcinogen causes a delay in the onset of regeneration and the parenchyma shows little change or perhaps some degeneration round the portal areas. There is a proliferation of connective tissue and bile ducts cells in the same region. The extent of the delay in regeneration naturally differs somewhat from animal to animal but there is in most cases a complete inhibition of regeneration for about five days and this may continue in a few animals for as long as fourteen days.

After this delay the second phase of regeneration starts in a few centres, usually near the central veins, and proceeds by enlargement of these centres to form multiple nodules which grow until they coalesce. The majority of the original parenchymal cells disappear either during the phase of inhibition, or during the enlargement of the nodules which finally replace them. Occasionally a liver may show slight signs of normal regenerative activity before nodule formation begins. The variations from animal to animal in the exact timing of these phases means that there is some variation in cultural behaviour, particularly in the earlier period up to seven days, but the general lines are clear cut. Specimens of liver from animals killed at seven and ten days after hepatectomy were also examined under the phase contrast microscope. It was found that the parenchyma could easily be split up into small masses of cells, probably representing the nodules seen in sections. These were not closely bound to each other by connective tissue fibres. Within the masses a few fibres were in evidence, but the most obvious change was in the adhesion of the cells to each other. These could be separated into groups of a few cells and even into individual cells with a little manipulation, in contrast to the behaviour of normal liver in which the cells adhere so closely that they disintegrate rather than separate. Treatment of such liver with aniline

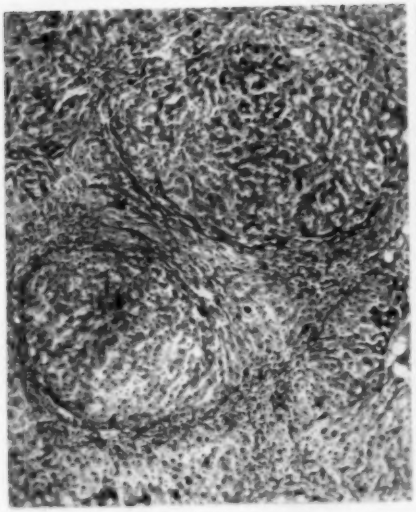
EXPLANATION OF PLATE

FIG. 1.—Section of liver of rat 14 days after partial hepatectomy, having received carcinogen three weeks before operation and subsequently until death. Nodules enlarging and compressing remnants of original tissue. H. and E. ×55. (Laws, 1949, Fig. 4.)

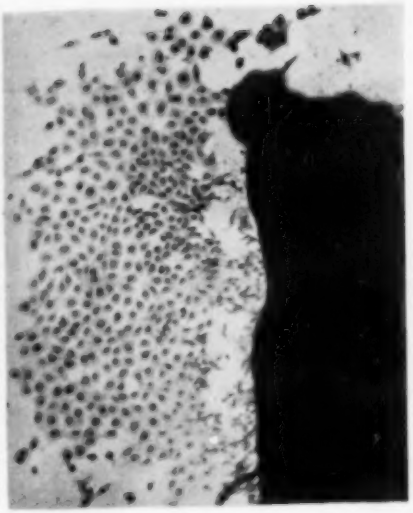
FIG. 2.—Stained culture from normal rat, explanted 24 hours after partial hepatectomy, 4 days *in vitro*. Epithelial sheet. Giemsa. ×50.

FIG. 3.—Stained culture from carcinogen-treated rat, explanted 10 days after partial hepatectomy, 8 days *in vitro*. Migration of individual cells, nuclei only stained. Giemsa. ×50.

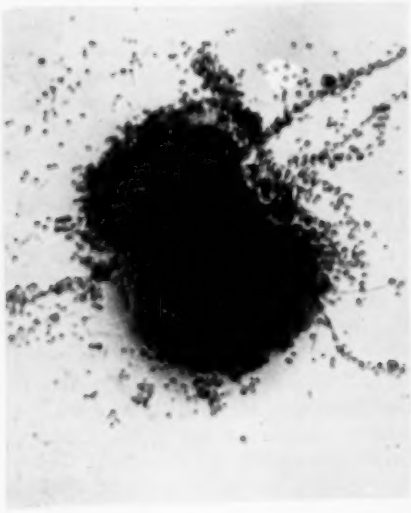
FIG. 4.—Stained culture from carcinogen-treated rat, explanted 14 days after partial hepatectomy, 13 days *in vitro*. Sparser migration of large individual cells, cytoplasmic granules stained. PAS. ×50.



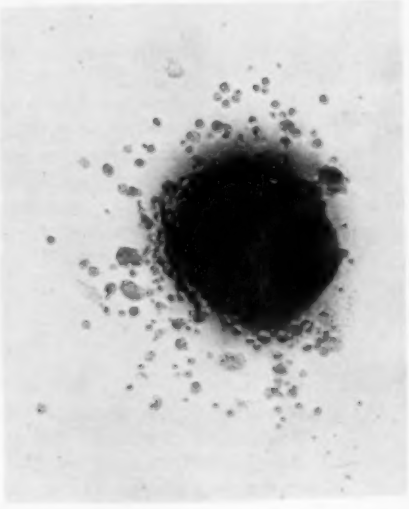
1



2



3



4

blue (Laws, 1961) showed the usual blue staining of the cell boundaries even when the cells were separated.

In the series explanted three days after hepatectomy, only one of the three animals used showed migration from the explants and histological examination showed that this alone was regenerating to a slight extent in a normal pattern. The outgrowth was largely of sheets of epithelium from which a few isolated cells broke off after a time. In the one rat killed at five days migration was active and showed first small sheets of epithelial cells followed up by a more massive migration of small groups and isolated cells which moved out further. Histological examination showed the beginnings of nodular regeneration.

The explants taken after seven, ten and fourteen days may be considered together as they followed a common pattern. Migration occurred in a proportion of the explants from all the animals killed in these groups, in an approximately equal distribution between individuals. Histologically all these animals showed nodular regeneration of the type described, in most cases the outgrowth began with the appearance of macrophages and fibroblasts although these were scarce or absent in some ten and fourteen day livers. These were rapidly succeeded by a vigorous outgrowth of isolated liver cells, which migrated for a considerable distance, up to 3–4 mm. in the ten-day livers which showed the maximum activity in this respect. The most rapid onset of epithelial outgrowth was however, seen in one of the fourteen day animals in which cells of this type were migrating as early as eighteen hours. These cells were large and granular and did not flatten out on the glass surface completely. The nuclear pattern was that of liver cells and they were invariably full of granules. The migration was vigorous, and where fibres of cotton wool were on occasion found in contact with explants, migration occurred along these sometimes from the top of the explant right down to the glass surface. In smaller explants consisting of nodular regenerate, which occurred particularly in the ten and fourteen day groups in which the nodules had practically coalesced, the migration thinned the explants considerably so that they were much reduced in size, after seven to ten days. Daily observations suggested that many cells in fact become dislodged from the glass in these circumstances. In none of these explants were the normal sheets of epithelium seen at any time. The cultures remained healthy rather longer than those from normal liver, often not degenerating until after fourteen days.

DISCUSSION

These results show a marked difference in the type of outgrowth seen under these particular conditions between explants from normal livers, whether young or adult and from the carcinogen-treated livers. Parenchymal migration on the normal livers occurred in sheets of flattened but still differentiated cells. Following carcinogen treatment the cells migrated as individual cells, considerably more rounded, although still adhering to the glass or other surface which is presented, and very active in their movement. They still retained their liver-cell characteristics, being particularly rich in glycogen granules.

In the normal group it is noteworthy that the adult rats showed less active parenchymal migration after hepatectomy than before. This appears in contrast to the findings of Glinos and Bartlett (1951) but it must be noted that these authors only reported quantitatively on migration as a whole, not distinguishing

simple macrophage-fibroblast outgrowth from epithelial cell migration. Moreover their explants were made on plasma clot, using a rich medium containing embryo extract and placental cord serum. Under such conditions epithelial cell migration is retarded but the activity of the connective tissue elements is enhanced.

The effect of the carcinogen treatment seems to be to facilitate epithelial migration and to inhibit the normal adhesion of the cells which is displayed in flattening and sheet formation. It would appear that two factors might be involved in this process. Firstly, the migration might be facilitated by a lessened binding of the organ by connective tissue fibres. In normal liver the level of collagen has been shown by Harkness (1958) to be reduced during the early stages of regeneration. This does not however in the present experiments appear to have led to any increased migration in normal livers, rather the contrary, so this is probably at the most a minor factor in the carcinogen-treated explants. The second factor is probably a change in the cell surface, since all the alterations in behaviour are consistent with such a hypothesis. Moreover the direct observation of the livers has shown changes in the intercellular adhesion. The nature of the change is not certain but probably involved the aniline-blue staining cell wall material, the role of which has been discussed in another paper (Laws, 1961). Whether this is the only change, and whether such a change in the carcinogen-treated livers is in any way related to similar changes seen in established tumours is at present unknown. It is perhaps relevant that Trevan and Roberts (1960, personal communication) have shown recently that ascites tumour cells growing normally as isolated cells may be induced under certain circumstances to form sheets in tissue culture, a process which is reversible. It seems possible that similar changes, but operating in the reverse direction, may be produced by the action of carcinogen *in vivo*.

The importance of these cell surface changes in the process of carcinogenesis is at present speculative. It has been shown (Laws, 1959) that partial hepatectomy of rats within the first few weeks of carcinogen feeding leads to the precocious appearance of hepatomas. Laird and Barton (1959) have suggested that the critical phase for initiating the process of carcinogenesis occurs at the onset of hyperplasia, which in the partially hepatectomised animal is at the onset of regeneration, that is the period under investigation in the present work. Haddow (1938) suggested that a period of growth inhibition followed by renewed cell division may produce suitable conditions for the primary carcinogenic change.

In the present experiments there is an inhibition of regeneration followed by rapid cell division. The physical isolation of the cells during the regeneration phase, as suggested by the results presented here, may also play a part in producing suitable conditions for the induction of carcinogenesis.

SUMMARY

(1) Liver regeneration has been induced by partial hepatectomy in rats fed with the carcinogen 2-acetylaminofluorene. At intervals following this procedure portions of the regenerating liver have been explanted into tissue culture and observed. Control explants have been made from three-week old rats and from adult rats before and at intervals after partial hepatectomy.

(2) The explants from all the rats untreated with carcinogen have given rise

to migration first of non-parenchymal cells and then to sheets of large epithelial cells having the characters of liver parenchyma. These sheets have persisted for a period of about ten days and then degenerated.

(3) The explants from carcinogen-treated livers behave in one of two general ways depending on the period which has elapsed following the partial hepatectomy. During the first five days, on the average, the liver histologically shows little sign of regenerative activity. During this period explants show little epithelial migration but occasionally small sheets of cells were seen together with a few large isolated cells.

Subsequently the liver shows vigorous regeneration of an abnormal nodular type. Explants at this stage give rise to the migration of individual large parenchymal cells which appear precociously and migrate actively over considerable distances on any support which is present. No epithelial sheets are to be seen and migration of non-parenchymal cells is minimal.

I would like to express my thanks to the Trustees of the Will Trust set up by the late Mrs. M. O. Gordon for the support which they have given to this work.

REFERENCES

EHRMANN, R. L. AND GEY, G. O.—(1956) *J. nat. Cancer Inst.*, **16**, 1375.
GLINOS, A. D. AND BARTLETT, E. G.—(1951) *Cancer Res.*, **11**, 164.
HADDOW, A.—(1938) *Acta Un. int. Cancr.*, **3**, 342.
HARKNESS, R. D.—(1958) 'Symposium on Liver Function.' Publication No. 4. Amer. Inst. biol. Sci. Washington D.C., p. 59.
HIGGINS, G. M. AND ANDERSON, R. M.—(1931) *Arch. Path.*, **12**, 186.
LAIRD, A. K. AND BARTON, A. D.—(1959) *Nature, Lond.*, **183**, 1655.
LAWS, J. O.—(1959) *Brit. J. Cancer*, **13**, 669.—(1961) *Exp. Cell. Res.*, in press.
Idem, MABILLE, P., ROYER, R. AND RUDALI, G.—(1952) *Bull. Ass. franç Cancer*, **39**, 450.
SKORYNA, S. C. AND WEBSTER, D. R.—(1951) *Proc. Soc. Exp. Biol. N.Y.*, **78**, 62.

QUANTITATIVE CYTOLOGICAL STUDIES ON THYMIC LYMPHOID CELLS IN NORMAL, PRELEUKAEMIC AND LEUKAEMIC MICE

K. NAKAMURA AND D. METCALF

From the Cancer Research Laboratory, Walter and Eliza Hall Institute, Royal Melbourne Hospital, Melbourne, Victoria, Australia

Received for publication February 1, 1961

THE incidence of spontaneous thymic lymphomata, with generalised lymphoid leukaemia, in AKR mice varies from 85–95 per cent. The first histological and biological evidence of leukaemia is usually to be found in the thymus, and the disease is commonly limited to the thymus (Furth and Boon, 1945). The first deaths from this disease usually occur in female mice at six months, and in male mice at seven months of age.

The AKR thymus therefore presents an ideal opportunity for a detailed cytological examination of the preleukaemic state. In the sense used in this paper, " preleukaemic " refers merely to AKR mice in the 0–7 months age period.

This paper presents the results of a comparative survey of AKR and C3H thymuses in this age period.

MATERIALS AND METHODS

Mice

Mice used were inbred males and females of strains AKR and C3H. The AKR mice (current lymphoid leukaemia incidence 90 per cent) were originally obtained from Dr. Jacob Furth. The C3H mice (current lymphoid leukaemia incidence 1–5 per cent) originated in the Imperial Cancer Research Fund Laboratories, London.

The mice were housed in metal tins, with sawdust bedding. The diet consisted of purina chow and water *ad lib.*, with carrots twice weekly and occasional greens. The room temperature of the animal colonies was maintained at 75° F. The AKR and C3H colonies were housed in separate, but adjacent animal rooms.

Cytological preparations

Cytological preparations of thymus cells were always made between 2 and 4 p.m. Mice were killed with ether, and the thymuses removed and weighed. The thymuses were then placed in separate Petri dishes, and 0·25 ml. saline added. The thymuses were finely minced with sharp curved scissors. An equal volume of glacial acetic acid/orcein was added and the mince mashed into minute fragments with angulated forceps. Acetic acid/gentian violet was then added (final concentration, 2 parts acetic acid/orcein : 1 part acetic acid/gentian violet).

Several drops of liquid glycerine were added to the mince, and a drop of the mince was placed on a slide. A cover slip was then gently lowered on the drop and pressed down on the slide until adequate cell spreading was obtained. The preparations were sealed with balsam/paraffin, and stored at 4° C.

Sampling technique

In these preparations, segregation of cell types occurred when the cover slip was lowered on the drop. The preparations were therefore sampled in a fixed battlement pattern, passing from one end of the cover slip to the other.

In each preparation, 5000 consecutive lymphocytes and 100 consecutive mitoses were typed.

Cell typing

The general appearance of these thymus preparations is shown in Fig. 1 and 2. In these preparations, the size of the lymphoid cells was to a large degree determined by the degree of spreading occurring during their preparation. Accordingly, lymphoid cells were typed on the basis of nuclear detail, little attention being paid to the apparent size of the cells. Fig. 3 shows the nuclear details of the cell types found in these preparations.

The epithelial/reticular cells were very few in number, relative to the number of lymphoid cells, and were not counted in this survey.

Mitotic classification

Only cells showing distinct chromosomes were scored as mitoses, cells in early prophase were not included in the mitotic count.

Mitoses classified as ? normal were cells in which the mitotic figure was not clearly visible. Cells in which it was doubtful whether the nucleus showed intense clumping of chromosomes or was merely pyknotic, were classified as ? pyknotic.

Statistical analysis

Statistical analyses were made using the Student "*t*" series method.

RESULTS

Thymuses were examined from male and female AKR mice of various ages—1, 3, 8, 16, 24 and 28 weeks. For comparison, thymuses were examined from male and female C3H mice of the same ages. The thymus weights of the mice examined (Tables I and II) were within the normal limits previously described for these strains (Metcalf, 1960).

Cell types

Table I shows the mean percentages of the various lymphoid cell types in male AKR and C3H thymuses of various ages. Significant strain differences were found in the percentages of medium and small lymphocytes.

In C3H mice, the percentage of medium lymphocytes fell progressively with increasing age. In AKR mice, the percentage of this cell type was significantly higher than in C3H mice. After an initial fall between 1 and 24 weeks, the percentage rose in the immediate preleukaemic period (24–28 weeks). Considerable individual variation (from 5·4–12·5 per cent) was encountered in the medium lymphocyte content of 28 week AKR thymuses.

The percentage of small lymphocytes in AKR thymuses was lower than in C3H thymuses, at all ages.

The survey of female AKR and C3H thymuses (Table II) revealed similar strain differences to those described in the male mice. Again there was a rise in the percentage of medium lymphocytes in AKR thymuses at 28 weeks, and there was considerable individual variation (from 8·4–14·8 per cent) in the medium lymphocyte content of 24 and 28 week old AKR thymuses.

Mitotic indices

The overall mitotic index (mitoses per 100 thymic cells) was maximal in male and female C3H thymuses at one week (Tables I and II), and fell sharply at the onset of age involution, between three and eight weeks. After this time, the mitotic index remained relatively constant.

The mitotic index of AKR thymic cells, at all ages, was higher than that of corresponding C3H cells. As in C3H thymuses, a fall in the index occurred at age involution. In the female AKR thymuses, this fall was delayed until after eight weeks. After 16 weeks, mitotic indices rose in AKR thymic cells, in both sexes, so that by 28 weeks, the indices averaged twice those of corresponding C3H cells. Again, considerable individual variation was encountered in AKR thymuses in the 28 week age group, values ranging from 0·30–1·12 per cent.

It will be noted that, at all ages, female AKR mitotic indices were consistently higher than corresponding male indices.

Mitotic abnormalities

A high frequency of abnormal mitoses was encountered in these thymus preparations. The most common abnormalities, in order of frequency were, (*a*) metaphase arrest, with chromosome swelling and stickiness, (*b*) failure of cytoplasmic division, (*c*) chromosome bridges and (*d*) chromosome clumping (Fig. 4).

The percentage incidence of mitotic abnormalities in AKR and C3H thymuses at various ages is shown in Tables I and II. In C3H thymuses, the percentage frequency of abnormal mitoses rose sharply after age involution, reaching levels of 60 per cent. A similar change was found in AKR thymuses, but the frequency of abnormal mitoses never exceeded 40 per cent, and fell after 24 weeks.

However, calculation of the absolute number of abnormal mitoses per 100 thymic cells (Table III) indicated that there was no strain difference in their incidence, and that there was little fluctuation with age. This was in contrast to the fluctuations in the absolute number of normal mitoses present in these thymuses. For example, in male mice, the number of normal mitoses per 100 cells in one week old AKR mice was 0·90, whilst the number in 28 week old C3H

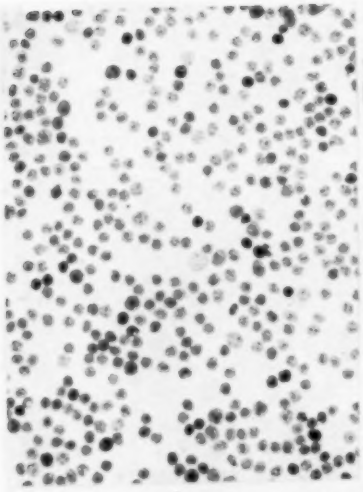
EXPLANATION OF PLATE

FIG. 1.—General view of cell preparation from normal six-months AKR thymus. Note absence of damaged cells. ×220.

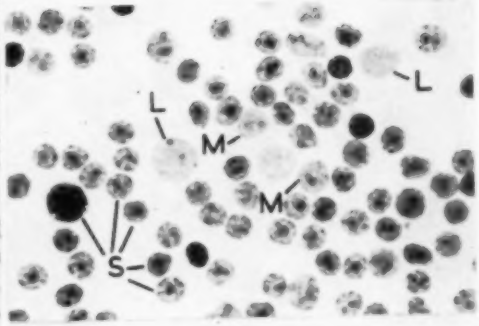
FIG. 2.—General view of cell preparation from AKR thymic lymphoma. Note preponderance of primitive cells. ×220.

FIG. 3.—Nuclear details of the various types of lymphoid cells from a normal six month AKR thymus. ×560. L, large lymphocyte. M, medium lymphocyte. S, small lymphocyte.

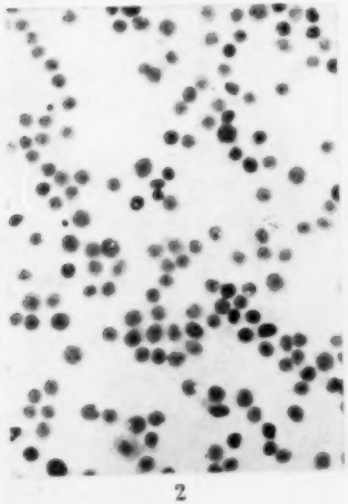
FIG. 4.—Mitotic abnormalities in normal thymic lymphoid cells. A and B chromosome bridges. C and D Metaphase arrest, with swelling and stickiness of chromosomes. E Failure of cytoplasmic division at telophase. F Binucleate cell. ×1000.



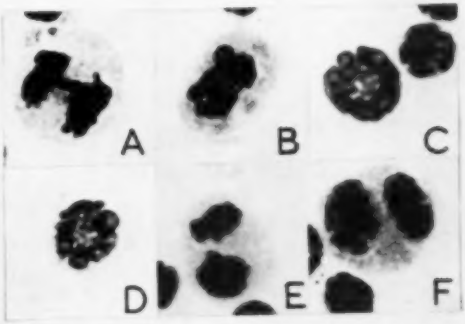
1



3



2



4

TABLE I.—*Thymus Cytology Male AKR and C3H Mice*

Strain	Age (weeks)	Number of thymuses	Mean thymus weight † (mg.)	Mean % large lympho-cytes	Mean % medium lympho-cytes	Mean % small lympho-cytes	Mean % mitoses	Mean % pyknotic cells	Per cent mitoses			
									Normal	? Normal	Ab-normal	? Pyknotic
AKR	1	6	24±5	1·5±0·6	11·1±0·7*	85·5±0·5*	1·1±0·1*	0·6±0·2	76±5	12±2	11±3	1±2
C3H	1	6	23±1	1·5±0·5	8·3±1·8	88·6±1·7	0·9±0·1	0·7±0·3	73±3	12±2	13±3	2±3
AKR	3	8	76±12*	1·4±0·3	9·9±1·9	87·0±1·7	1·0±0·1*	0·8±0·2	72±10	12±2	13±5	3±3
C3H	3	6	54±12	1·5±0·4	8·3±0·4	88·6±0·2	0·7±0·1	0·8±0·2	66±5	13±2	17±3	4±3
AKR	8	6	105±8 *	2·2±0·8	9·5±1·0	86·9±1·1*	0·6±0·2	0·9±0·3	58±12*	14±3	19±3	9±7
C3H	8	6	50±12	1·7±0·3	7·6±1·3	89·6±1·5	0·4±0·1	0·8±0·2	35±6	18±2	32±4	15±6
AKR	16	6	73±7 *	2·3±0·6	8·3±0·9	88·1±0·9	0·5±0·1*	0·8±0·1	55±7 *	12±3	26±4	7±2
C3H	16	6	28±8	1·7±0·4	6·7±1·1	89·1±2·0	0·4±0·1	0·6±0·3	36±6	12±2	35±8	17±4
AKR	24	10	46±7 *	1·8±0·4	8·2±1·2*	88·4±1·4*	0·7±0·1*	0·9±0·2	56±15*	12±3	23±9	9±6
C3H	24	14	25±7	1·5±0·4	6·1±1·2	91·2±1·6	0·4±0·2	0·8±0·4	37±7	13±2	36±7	14±3
AKR	28	8	52±23*	2·3±0·6	9·1±2·5*	87·2±2·9*	0·7±0·3*	0·8±0·2	72±9*	11±3	13±5	4±6
C3H	28	6	21±4	1·8±0·2	5·5±1·4	91·7±1·8	0·3±0·1	0·7±0·2	31±5	15±2	36±3	18±3

† ±Standard deviation. * Results significantly different $P<0.01$

TABLE II.—*Thymus Cytology Female AKR and C3H Mice*

Strain	Age (weeks)	Number of thymuses	Mean† thymus weight (mg.)	Mean % large lympho-cytes	Mean % medium lympho-cytes	Mean % small lympho-cytes	Mean % mitoses	Mean % pyknotic cells	Per cent mitoses			
									Normal	? Normal	Ab-normal	? Pyknotic
AKR	1	6	23±6	1·7±0·1	11·3±0·4	84·8±0·4	1·4±0·1	0·8±0·1	76±2	12±1	11±2	1±1
C3H	1	6	12±2	1·4±0·3	11·0±0·6	85·5±0·6	1·2±0·1	0·9±0·1	72±4	13±1	13±2	2±2
AKR	3	6	87±3 *	1·2±0·2	10·3±0·4	86·6±0·6	1·1±0·1	0·9±0·1	70±4	15±3	13±3	2±1
C3H	3	6	56±11	1·1±0·2	9·6±0·5	87·2±0·5	0·9±0·1	0·9±0·2	63±5	14±1	18±3	5±3
AKR	8	6	129±13*	1·4±0·4	9·0±1·1	87·9±0·9	0·9±0·1*	0·9±0·2	69±5*	12±1	16±7	3±2
C3H	8	8	65±10	1·7±0·4	7·7±0·9	89·1±0·8	0·6±0·1	0·9±0·3	50±8	16±6	27±6	7±4
AKR	16	6	96±18*	1·4±0·1	8·4±0·7	88·6±0·6*	0·7±0·1*	1·0±0·2	51±6*	16±2	27±5	6±2
C3H	16	7	44±4	1·4±0·3	7·3±0·6	90·1±0·5	0·5±0·1	0·8±0·2	34±4	14±2	40±4	12±3
AKR	24	9	99±15*	1·5±0·5	10·2±1·7	86·6±2·0	0·9±0·1*	0·9±0·2	61±7*	14±1	21±5	4±3
C3H	24	7	36±9	1·2±0·1	8·4±0·6	88·8±0·6	0·5±0·1	1·0±0·1	43±8	15±2	33±6	9±3
AKR	28	7	83±23*	1·5±0·4	10·3±1·2*	86·2±2·0*	0·9±0·2*	0·9±0·2	70±5*	13±1	14±3	3±2
C3H	28	8	23±5	1·3±0·1	8·0±0·6	89·0±0·7	0·5±0·1	1·0±0·1	46±8	17±2	30±5	7±3

† ±Standard deviation. * Results significantly different $P<0.01$.

TABLE III.—*Absolute Number of Abnormal Mitoses per 100 Thymic Cells*

Strain	Sex	Age (weeks) 1	3	8	16	24	28
AKR	M.	0·20	0·21	0·19	0·21	0·26	0·14
C3H	,,	0·18	0·20	0·21	0·20	0·23	0·19
AKR	F.	0·25	0·25	0·22	0·28	0·27	0·25
C3H	,,	0·27	0·28	0·24	0·29	0·25	0·25

mice was 0·11 (an eight-fold variation), yet the abnormal mitoses per 100 cells were respectively, 0·20 and 0·19.

Corrected mitotic indices of AKR and C3H thymic cells

The following figures (Fig. 5, 6) present the mean corrected mitotic indices with standard deviations, for AKR and C3H large and medium cells (combined) at the various ages studied. In these calculations, all mitoses seen have been assumed to be in either large or medium cells. Further, a correction factor has been applied on the basis that, the abnormal mitoses seen were ineffective in giving rise to functional offspring.

For example, if the overall mitotic index was 0·6 per cent, with 10 per cent large and medium cells, and a mitotic distribution of: normal 60 per cent, ? normal 10 per cent, abnormal 20 per cent and ? pyknotic 10 per cent, then the calculated corrected mitotic index is

$$\frac{0{\cdot}6 \times 100 \times 65}{10{\cdot}6 \times 100}$$

per cent of large and medium cells (half of ? normal mitoses are calculated as normal, half abnormal).

The calculations for both male and female mice indicate that, after the first few weeks of life, AKR primitive lymphoid cells exhibited mitotic indices which were approximately twice those of corresponding C3H cells.

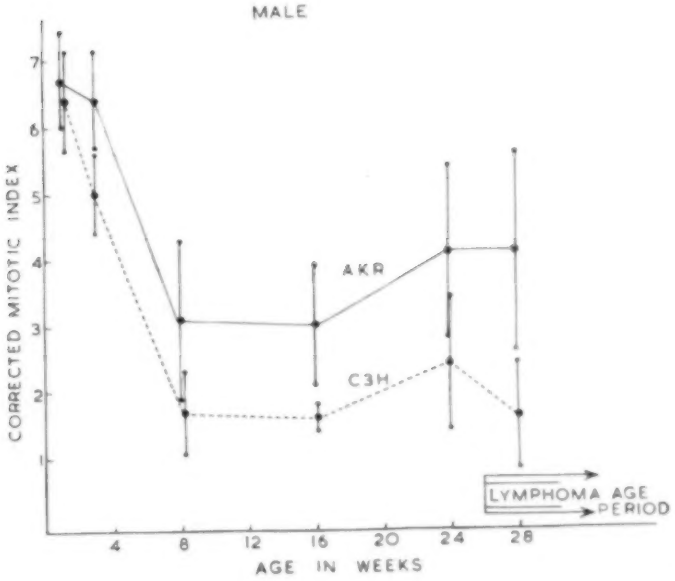
Mitotic times in AKR and C3H cells

Male AKR and C3H mice, aged 4 months, were injected I.P. with 80 gamma of colchicine. Three hours later, these mice, along with control mice, were killed and their thymuses examined cytologically.

In the colchicine-treated thymuses, no telophase or anaphase mitotic figures were seen, indicating that blockage of mitosis was complete in both strains. Table IV presents the results obtained on the accumulation of mitoses, following colchicine injection.

The accumulation rate was almost identical in AKR and C3H thymic lymphoid cells. On the assumption that both daughter cells of the dividing cells remained capable of division, the average time for the visible stages of mitosis approximated 35 minutes in both strains.

In both the AKR and C3H thymuses, the percentage of large lymphocytes was significantly lower in the colchicine-treated mice, than in the control mice suggesting that many of the arrested mitotic cells were large lymphocytes.



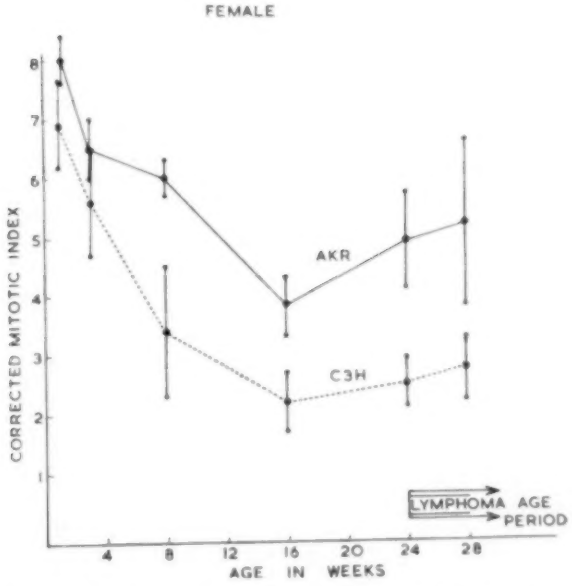


FIG. 5 and 6.—Corrected mitotic indices of primitive lymphoid cells in male and female AKR and C3H thymuses.

TABLE IV.—*Effect of Colchicine on Thymus Cytology*

Strain	Treatment	Number of mice	Thymus weight † (mg.)	Large lymphocytes (%)	Medium lymphocytes (%)	Small lymphocytes (%)	Mitoses (%)	Pyknoses (%)	Accumulation factor
AKR	Colchine 80γ 3 hours	5	59±7	1·3±0·1	6·4±0·1	86·8±0·9	3·08±0·60	2·4±0·7	5·7
AKR	—	6	73±7	2·3±0·6	8·3±0·9	88·1±0·9	0·54±0·14	0·8±0·1	—
C3H	Colchicine 80γ 3 hours	5	32±2	1·2±0·1	6·8±0·4	87·8±1·0	1·95±0·10	2·2±0·7	5·6
C3H	—	6	28±8	1·7±0·4	6·7±1·1	89·1±2·0	0·35±0·08	0·6±0·3	—

† ±Standard deviation.

TABLE V.—*AKR Spontaneous Thymic Lymphomata*

No.	Age (months)	Sex	Large lymphocytes (%)	Medium lymphocytes (%)	Small lymphocytes (%)	Mitoses (%)	Pyknoses (%)	Per cent mitoses				Corrected mitotic index
								Normal	? Normal	Abnormal	? Pyknotic	
1	10	M.	15·2	62·4	17·9	1·80	2·85	42	21	32	5	1·2
2	8	F.	21·9	53·9	15·7	1·95	6·60	43	23	29	5	1·3
3	9	M.	28·4	54·4	11·9	2·08	2·92	46	14	29	11	1·3
4	7	F.	34·9	52·2	7·5	2·34	3·12	30	18	33	19	1·0
5	8	M.	45·8	38·2	7·5	2·48	6·06	27	13	39	21	0·9
6	7	,,	22·0	54·6	18·6	1·30	3·50	—	—	—	—	1·1
7	9	,,	32·0	34·5	22·4	1·92	9·10	—	—	—	—	1·2
8	8	,,	3·7	47·6	46·2	1·45	1·20	55	21	21	4	1·7
9	8	,,	4·3	58·4	32·0	1·25	4·05	47	18	31	4	1·1
10	8	F.	10·1	52·5	33·1	0·90	3·40	66	14	18	2	1·0
Mean		—	21·8	50·9	21·3	1·75	4·28	45	18	29	8	1·2

Cell analysis of AKR spontaneous thymic lymphomata

For comparison with the preceding data on preleukaemic AKR mice, thymuses were analysed cytologically from ten AKR mice suffering from spontaneous lymphoid leukaemia, with large thymic lymphomata. The results obtained are listed in Table V.

Some difficulty was encountered in classifying the lymphoid cells because of the occurrence of intermediate forms between the three cell types. The percentage distribution of the various cell types recorded is therefore only approximate.

Mitotic abnormalities similar to those seen in normal thymuses were also encountered in these preparations. In addition, abnormalities typical of neoplastic cells were also seen, e.g. tripolar mitoses. Thus the four categories of mitotic types do not correspond exactly to those for the normal thymuses.

It is evident from the table that the AKR lymphoma tissue examined was composed mainly of primitive lymphoid cells. The overall mitotic index per 100 thymic cells was two to three times that seen in the preleukaemic AKR thymuses.

When the mitotic index was corrected for abnormal mitoses and related to the primitive cell population (large + medium cells), the mitotic index of approximately 1·2 per cent was obtained. This figure showed little variation from one lymphoma to another.

Thus two significant changes appeared to occur in the transition from the preleukaemic to the leukaemic state. These were the rapid accumulation of primitive cells (presumably neoplastic), and a sharp fall in their mitotic index from 4·0–5·0 per cent to 1·2 per cent.

In one seven month male AKR thymus in which an early lymphoma nodule was present in one lobe, the cellular composition and mitotic activity of the opposite normal lobe was typical for 28 week male AKR thymic tissue. No evidence was found of an atrophic state preceding lymphoma development.

DISCUSSION

The results have confirmed the earlier observations of Kindred (1940) and Andreasen and Christensen (1949) that the mouse thymus is an intensely active site of lymphopoiesis.

Recent data, obtained from the labelling of lymphoid cells with tritiated thymidine (Cronkite *et al.*, 1959; Schooley, Bryant and Kelly, 1959), suggest that small lymphocytes rarely, if ever, enter mitosis. If so, the mitoses seen in the present thymus preparations presumably were in large or medium lymphocytes. Contrary to the claims of Sainte-Marie and Leblond (1958) it is not possible to classify the type of a lymphoid cell, when that cell is in mitosis. We have therefore expressed the mitotic activity of the thymic cells as mitoses per large and medium cells (combined). The data would be of more value for an understanding of the preleukaemic state if some method could be developed, whereby individual mitotic indices could be assigned separately to the large and medium cell populations.

When the large and medium lymphocytes were considered as a group, their mitotic rate was found to be very high. These cells divided in C3H mice, on an average, every 24 hours, and in AKR mice, every 12 hours.

The sharp fall in proliferative activity of the thymic cells, in both strains, between three and eight weeks of age, is possibly the cytological basis for the

characteristic age involution weight loss of the thymus at this time, a phenomenon not previously explained (Dougherty, 1952). Supporting this conclusion was the delay in the fall of mitotic activity in female AKR mice. This corresponds closely to the delay in the onset of age involution in these mice, described previously (Metcalf, 1960).

The finding of a high percentage of abnormal mitoses in normal thymuses was unexpected. They appear unlikely to have been artifacts, or due to random faults in a certain percentage of mitoses, since there was no constant ratio between normal and abnormal mitoses. Similar mitotic abnormalities, though in lesser numbers, have been reported in normal liver cells by Brues and Reitz (1951).

It is possible that many of these mitoses were merely temporarily deranged, and may have been ultimately capable of producing viable daughter cells. However, the abnormalities closely resembled those studied by Levan (1954) and Hirono (1951). Both groups produced evidence to indicate that the cells resulting from such mitoses were abnormal, in that they either died prematurely or were unable to divide.

The survey of the high-leukaemia strain AKR mice showed that, from birth, thymus lymphocyte mitotic activity was significantly higher than in the low-leukaemia strain C3H mice. This was more pronounced after age involution, when mitotic indices of AKR thymic cells were approximately double those of C3H cells. In the period immediately preceding the leukaemic age period (24–28 weeks), mitotic activity rose even higher in AKR cells.

However, the weights of the AKR thymuses did not increase indefinitely after birth, but instead showed typical age involution weight losses. Further, for the first 24 weeks, the percentage of primitive cells showed no increase. From these facts, it can be concluded that, during the first 24 weeks, the excessive proliferation of primitive AKR cells was fully compensated by an increased rate of differentiation and cell destruction or utilisation.

The situation in the immediate preleukaemic period appeared to be significantly different. A further rise in the mitotic index occurred, but there was, in addition, a rise in the percentage of primitive cells. This rise was confined exclusively to the medium lymphocytes.

The nature of this short period of apparent failure of differentiation is not certain, but it is of great theoretical importance. The biological properties of the AKR thymic cells in this phase are being further investigated.

AKR lymphoma tissue was characterised by a high content of primitive cells, having a strikingly low mitotic index. The ability of lymphoma cells to form a population of cells which increases in size exponentially, appears to reside in their relative insensitivity to lymphocyte differentiation enforcing mechanisms. This allows the progressive accumulation of a population of cells, which remain primitive, and are therefore capable of further division, even though the actual division rate of these cells is relatively slow.

The origin of a population of incompletely differentiating lymphoid cells in the AKR thymus does not appear to be the result of a failure of lymphocyte differentiating control mechanisms. Differentiation was normal in the normal lobe of the thymus showing an early lymphoma nodule in the other lobe. Further, lymphomas may be passaged by cell grafting to form progressively growing transplanted tumors, in young adult animals, whose lymphocyte differentiating mechanisms are presumably normal.

It seems likely therefore that the altered status of differentiation is the result of an intrinsic change in certain of the thymic lymphoid cells themselves, rendering them relatively insensitive to the body differentiating control mechanisms.

The initial hyperplasia, and the subsequent failure of differentiation may be causally related since there is evidence that factors increasing lymphopoiesis also increase the incidence of lymphoid leukaemia, and *vice versa* (see review by Kaplan, 1954).

The rapid division rate of the primitive AKR lymphoid cells may lead to errors of replication, or render these cells sensitive to damage by extrinsic leukaemogenic agents, such as hormones, carcinogens or the Gross virus.

The findings in preleukaemic AKR mice reported here support the theoretical concepts of carcinogenesis as proposed by Berenblum (1954) for skin carcinogenesis and the recent postulates by Kaplan (1959) on the probable nature of the neoplastic change in mouse lymphoid leukaemia.

SUMMARY

A quantitative cytological study has been made of the thymus in male and female C3H and AKR mice, from birth to the age of twenty-eight weeks.

In both strains, the thymus cells showed a high mitotic activity, but many of the mitoses were abnormal.

The mitotic indices of AKR thymic cells were approximately twice those of corresponding C3H cells throughout this period. The mitotic time for these cells was the same in both strains.

In the immediate pre-leukaemic period (twenty-four to twenty-eight weeks), an accumulation of medium lymphocytes was observed in AKR thymuses.

AKR thymic lymphomata contained a high percentage of primitive cells with mitotic indices much lower than those of normal primitive cells.

This work was supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria.

REFERENCES

Andreasen, E. and Christensen, S.—(1949) *Anat. Rec.*, **103**, 401.
Berenblum, I.—(1954) *Cancer Res.*, **14**, 471.
Brues, A. M. and Reitz, L.—(1951) *Ann. N.Y. Acad. Sci.*, **51**, 1497.
Cronkite, E. P., Fleidner, T. M., Bond, V. P., Rubini, J. R., Breecher, G. and Quastler, H.—(1959) *Ibid.*, **77**, 803.
Dougherty, T. F.—(1952) *Physiol. Rev.*, **32**, 379.
Furth, J. and Boon, M. C.—(1945) *Amer. Ass. Advanc. Sci.*, Conference on Cancer, Washington, p. 129.
Hirono, I.—(1951) *Gann*, **42**, 225.
Kaplan, H. S.—(1954) *Cancer Res.*, **14**, 535.—(1959) in " Carcinogenesis " CIBA Symposium. London (Churchill), p. 233.
Kindred, J. E.—(1940) *Amer. J. Anat.*, **67**, 99.
Levan, A.—(1954) *Hereditas*, **15**, 1.
Metcalf, D.—(1960) *Cancer Res.*, **20**, 1347.
Sainte-Marie, G. and Leblond, C. P.—(1958) *Proc. Soc. exp. Biol., N.Y.*, **97**, 263.
Schooley, J. C., Bryant, B. J. and Kelly, L. S.—(1959) in ' Kinetics of Cellular Proliferation '. New York (Grune and Stratton), p. 208.

TRANSPLANTATION BIOASSAY OF THYMUSES FROM PRELEUKAEMIC AKR MICE FOR THE PRESENCE OF LEUKAEMIC CELLS

D. METCALF AND K. NAKAMURA

From the Cancer Research Laboratory, Walter and Eliza Hall Institute, Royal Melbourne Hospital, Melbourne, Australia

Received for publication February 1, 1961

A QUANTITATIVE study has previously been reported (Nakamura and Metcalf, 1961) of the lymphocyte population in the thymuses of mice of high-leukaemia strain AKR, from birth to the development of thymic lymphomata.

It was noted that, in the period immediately preceding the appearance of thymic lymphomata (24-28 weeks), the AKR thymus contained elevated numbers of primitive cells.

The possibility existed that the elevated percentage of primitive cells in these thymuses at this time might have been due to the presence in varying numbers of fully-developed leukaemic cells.

It has previously been shown by Furth and Boon (1945), and more recently by Latarjet and co-workers (Latarjet, 1959) that isotransplantation of AKR lymphoid tissues can detect the presence of very small numbers of leukaemic cells.

In an attempt to analyse the nature of the changes in AKR thymuses in the immediate pre-leukaemic period, groups of seven month male, and six and seven month female thymuses were examined cytologically. These thymuses were simultaneously tested, by transplantation, for the presence of leukaemic cells.

MATERIALS AND METHODS

Mice

The mice used were AKR mice from the inbred colony maintained in this Institute. These mice were originally obtained from Dr. Jacob Furth, and have a current incidence of spontaneous leukaemia of 90 per cent.

The mice were housed in metal tins, with sawdust bedding. They were fed purina chow and water *ad lib.*, with carrots twice weekly and occasional greens.

The room temperature of the animal room was maintained at 75° F.

Cytology and transplantation

Six and seven month old AKR mice were selected at random from the inbred colony.

The thymus was removed sterilely, from each mouse, and half of each lobe cut free and prepared for cytological examination. These thymus preparations were wet-mounted suspensions of acetic acid-fixed cells, stained with orcein and gentian violet. The technique used has been described in detail elsewhere (Nakamura and Metcalf, 1961). Five thousand cells, and one hundred mitoses in each thymus preparation were classified, according to the criteria previously

described. The thymic lymphoid cells were classified as large, medium or small, and the incidence of abnormal mitoses was recorded. As previously described (Nakamura and Metcalf, 1961), the corrected mitotic index was calculated on the number of normal mitoses per large and medium lymphocytes (combined).

The other half of each lobe, together with the spleen from each animal was minced in chilled sterile saline with fine curved scissors.

The total minced material was then injected intramuscularly into the right thighs of four recipient AKR mice of the same sex. The age of the recipient mice at the time of transplantation was six weeks.

The transplanted mice were observed for three months. At this time surviving mice were killed and examined.

RESULTS

The cytological data on the thymuses examined have been arranged in three groups, on the basis of the results of transplantation of the thymus/spleen minces.

One of three types of result occurred in the recipient mice following transplantation :—

(*a*) thigh tumours with generalised non-thymic lymphoid leukaemia, or generalised leukaemia with preponderance of tumour growth in the lymph nodes draining the injection site ;

(*b*) no evidence of leukaemia ;

(*c*) thymic lymphomata development with no evidence of thigh tumours or involvement of lymph nodes draining the injection site.

Six of the twenty-three thymus/spleen minces tested produced thigh tumours, with generalised leukaemia in all but two of the recipient mice. The results of the cytological analysis of the six thymuses used as donor material are listed in Table I. Two of the thymuses (No. 4 and 5) showed elevated percentages of large and medium lymphocytes, and one (No. 1) an elevated percentage of large lymphocytes. The other thymuses had cytological patterns typical of AKR thymuses of this age (Nakamura and Metcalf, 1961).

Seven of the thymus/spleen minces produced no evidence of leukaemia in the recipient mice during the three month observation period. The cytological details of the donor thymuses in this group are listed in Table II. One of the thymuses (No. 10) contained elevated percentages of large and medium lymphocytes. The mean percentages of the various cell types for this group of seven thymuses were typical of AKR thymuses of this age. Only one thymus (No. 7) had a low content of primitive cells.

Ten of the thymus/spleen minces produced no evidence of thigh tumours or transplanted leukaemia in the draining lymph nodes. However, in 15 of the 39 recipient mice, thymic lymphomata developed, late in the three month observation period. The pathology of these tumours was identical with that of the spontaneous disease in AKR mice. In two of the mice, early involvement of other lymphoid organs was noted.

The cytological details of the ten thymuses in this group are shown in Table III. Three of the ten (No. 20, 22 and 23) showed elevated percentages of medium lymphocytes, and the mean figures for the whole group were typical of AKR thymuses of this age. In none of the thymuses in this group was the cytology suggestive of lymphoma tissue.

TABLE I.—*Analysis of Thymuses Producing Transplanted Leukaemia on Grafting*

Mouse No.	Age (months)	Sex	Thymus weight (mg.)	Large lymphocytes (%)	Medium lymphocytes (%)	Small lymphocytes (%)	Mitoses (%)	Pyknotic cells (%)	Per cent mitoses: Normal	? Normal	Abnormal	? Pyknotic	Corrected mitotic index	Number of transplanted leukaemias in recipient mice
1	6	M.	55	3·4	7·0	88·5	0·8	0·4	—	—	—	—	—	4/4
2	7	,,	35	1·3	12·0	85·9	0·2	0·6	64	13	16	17	1·1	2/4
3	7	F.	110	1·0	9·9	87·5	1·0	0·7	71	12	15	2	6·2	4/4
4	7	,,	110	3·2	31·7	61·9	1·4	1·8	83	6	10	1	3·3	4/4
5	7	,,	110	3·2	19·6	75·2	1·0	1·0	80	7	11	2	3·6	4/4
6	7	,,	70	1·5	12·0	84·2	1·1	1·2	71	12	15	2	5·8	4/4
Mean	—	—	—	2·3	15·4	80·5	0·9	0·9	74	10	13	3	4·0	—

TABLE II.—*Analysis of Thymuses Producing No Leukaemia on Grafting*

Mouse No.	Age (months)	Sex	Thymus weight (mg.)	Large lymphocytes (%)	Medium lymphocytes (%)	Small lymphocytes (%)	Mitoses (%)	Pyknotic cells (%)	Per cent mitoses: Normal	? Normal	Abnormal	Pyknotic	Corrected mitotic index	Number of leukaemias in recipient mice
7	7	M.	25	1·6	5·4	91·9	0·5	0·7	81	11	7	1	4·1	0/4
8	7	,,	85	2·6	7·8	88·0	0·8	0·9	69	12	15	4	5·7	0/4
9	7	,,	85	2·0	9·4	87·0	0·8	0·9	66	15	12	7	5·6	0/4
10	7	,,	45	2·9	12·5	82·5	1·1	0·9	75	6	13	6	8·7	0/4
11	6	F.	70	2·3	7·3	89·4	0·4	0·6	—	—	—	—	—	0/4
12	6	,,	—	1·5	9·8	87·0	0·9	0·7	66	12	19	13	5·5	0/4
13	7	,,	—	1·3	9·6	87·1	0·9	1·1	74	12	13	1	6·0	0/4
Mean	—	—	—	2·0	8·8	87·6	0·8	0·8	71	11	3	5	5·9	—

TABLE III.—*Analysis of Thymuses Producing Thymic Lymphomata on Grafting*

Mouse No.	Age (months)	Sex	Thymus weight (mg.)	Large lymphocytes (%)	Medium lymphocytes (%)	Small lymphocytes (%)	Mitoses (%)	Pyknotic cells (%)	Per cent mitoses: Normal	? Normal	Abnormal	Pyknotic	Corrected mitotic index	Number of thymic lymphomata in recipient mice
14	7	M.	55	2·1	8·2	88·6	0·6	0·5	83	8	7	2	5·2	1/4
15	7	,,	40	2·7	6·6	89·7	0·3	0·7	78	8	11	3	2·5	1/4
16	6	F.	—	1·2	8·9	87·9	0·7	1·2	69	13	15	3	4·8	3/4
17	6	,,	75	2·3	9·4	87·3	0·5	0·4	—	—	—	—	—	3/4
18	6	,,	—	1·1	9·0	88·1	0·8	1·0	59	15	23	3	5·0	1/4
19	6	,,	—	1·3	9·7	87·5	0·8	0·7	63	15	21	1	4·7	1/4
20	7	,,	—	2·2	11·4	84·4	1·0	1·0	61	14	20	5	4·6	1/3
21	7	,,	80	1·2	8·4	89·0	0·5	0·9	64	12	11	3	3·3	1/4
22	7	,,	40	1·9	10·7	86·0	0·6	0·8	70	15	12	3	3·4	1/4
23	7	,,	120	1·2	11·4	85·4	1·2	0·8	76	11	13	0	6·9	2/4
Mean	—	—	—	1·7	9·4	87·4	0·7	0·8	69	13	15	3	4·5	—

The incidence of spontaneous thymic lymphomata in young AKR mice was checked by killing and examining groups of male and female AKR mice. No thymic lymphomata were found in 55, five months old male mice. In 36, 5–5½ months old female AKR mice, only one early lymphoma was found—this in a 5½ months old mouse. Thus the occurrence of 15 thymic lymphomata in 39 4½ months old mice, in the present experiment, is unlikely to have been due to spontaneous leukaemogenesis.

Table IV presents an analysis of the results produced in the recipient mice, according to the age and sex of the donor mice. It may be seen that negative results were obtained most commonly with 6 month male tissues, transplanted tumours with 7 month female tissues. Both the 6 and 7 month female tissues commonly produced thymic lymphomata in the recipient mice. Thus, an age gradient in the response to transplantation appeared to exist from no tumour production, to thymic lymphoma production, to production of transplanted leukaemia.

TABLE IV.—*Summary of Results Produced by Injected AKR Cells*

Age and sex of donor mice	Number of donor samples tested	Effects in recipient mice*		
		Nil	Thymic lymphomata	Transplanted leukaemia
7 month ♂	7	4	2	1
6 month ♀	6	2	4	—
7 month ♀	9	1	4	4

* 4 Recipient mice injected with each tissue mince.

DISCUSSION

The primary object of these experiments was to investigate the nature of the increased percentage of primitive cells in the thymuses of AKR mice in the age period immediately preceding the appearance of spontaneous thymic lymphomata.

Of the twenty-three thymuses tested, less than a third contained transplantable leukaemic cells, although all but one showed increased percentages of primitive cells.

These results suggest that in many six and seven month AKR thymuses, the elevated percentage of primitive cells is not due to the accumulation of leukaemic cells, but represents a phase of failure of differentiation of thymic lymphoid cells, immediately preceding the appearance of transplantable neoplastic cells.

The occurrence of such a phase in carcinogenesis has been proposed on theoretical grounds for skin carcinogenesis, by Berenblum (1954). The probable importance of failure of differentiation as a critical step in carcinogenesis has also been emphasised by Kaplan (1959) in the development of thymic lymphomata in mice following whole-body irradiation.

The appearance of thymic lymphomata in the recipients of ten of the twenty-three thymus/spleen minces was unexpected. It is possible that their incidence might have been higher had not the experiment been closed three months after transplantation. Three possible interpretations may be advanced to explain this finding :—

(*a*) acceleration of the development of spontaneous thymic lymphomata,
(*b*) induction of thymic lymphomata in the recipient mice, or

(*c*) seeding of transplanted cells in the recipients' thymuses, with progression of these cells to neoplasia.

Acceleration of lymphoma development in AKR mice has been described by Schwartz, Schoolman and Szanto (1955) and by Latarjet (1959) following the injection of whole cells or extracts from AKR tissues. The nature of this phenomenon is not clear.

True induction of lymphomata in the AKR recipient mice appears unlikely in view of the negative results of Gross (1954) and others (Dulaney *et al.*, 1957; Latarjet, 1959) in attempts to induce lymphomata in adult mice by injecting non-cellular material from unpassaged spontaneous lymphomata.

The third possibility has not received attention previously. It is now apparent from the work of Law (1952), Kaplan, Hirsch and Brown (1956), Barnes *et al.* (1959) and Miller (1960) that lymphoma development in mice is dependent on some non-cellular iufluence exerted by the thymus on the lymphoid cells. It is possible that in the six and seventh month AKR mouse, there are some lymphoid cells, which are not normal, yet not frankly neoplastic. If some of these cells, after transplantation, seed in the recipient's thymus and there are exposed to the thymic influence, progression to full neoplasia may occur in these cells, with the development of thymic lymphomata.

The above possibilities are being further investigated.

SUMMARY

Twenty-three thymuses from 6 and 7 month old AKR mice were cytologically analysed and tested for the presence of leukaemic cells by intramuscular transplantation into AKR recipients.

Although twenty-two of these thymuses showed elevated percentages of primitive cells, only six contained transplantable leukaemic cells. This suggests that failure of differentiation precedes the development of neoplasia in thymic cells.

Ten thymuses, on transplantation, produced no evidence of transplanted leukaemia, but produced thymic lymphomata in 15 of the 39 recipient mice, within three months of transplantation.

It is suggested that an initial thymus dependent phase may exist in lymphoma cells developing in AKR mice.

This work was supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria.

REFERENCES

Barnes, D. W. H., Ford, C. E., Ilbery, P. L. T., Jones, K. W. and Loutit, J. F.—(1959) *Acta Un. int. Cancr.*, **15**, 544.

Berenblum, I.—(1954) *Cancer Res.*, **14**, 471.

Dulaney, A. D., Maxey, M., Schilling, M. G. and Goss, M. F.—(1957) *Ibid.*, **17**, 809.

Furth, J. and Boon, M. C.—(1945) *Amer. Ass. Advanc. Sci.*, Conference on Cancer, Washington, p. 129.

Gross, L.—(1954) in 'Leukaemia Research' CIBA Symposium. London (Churchill), p. 76.

KAPLAN, H. S.—(1959) in 'Carcinogenesis' CIBA Symposium. London (Churchill), p. 233.
Idem, HIRSCH, B. B. AND BROWN, M. B.—(1956) *Cancer Res.*, **16**, 434.
LATARJET, R.—(1959) in 'Carcinogenesis' CIBA Symposium. London (Churchill), p. 274.
LAW, L. W.—(1952) *J. nat. Cancer Inst.*, **12**, 789.
MILLER, J. F. A. P.—(1960) *Brit. J. Cancer*, **14**, 93.
NAKAMURA, K. AND METCALF, D.—(1961) *Ibid.*, **15**, 306.
SCHWARTZ, S. O., SCHOOLMAN, H. M. AND SZANTO, P. B.—(1955) *J. Lab. clin. Med.*, **46**, 949.

THE OCCURRENCE OF MALIGNANT LYMPHOMAS IN URETHAN-TREATED SWISS MICE

B. TOTH, G. DELLA PORTA* AND P. SHUBIK

From the Division of Oncology, The Chicago Medical School, Chicago, Illinois, U.S.A.

Received for publication April 15, 1961

THE carcinogenic action of urethan (ethyl carbamate), once thought specific for pulmonary tissue (see review by Shimkin, 1955), has recently been found to extend to other tissues. Tannenbaum and Silverstone (1958) using repeated skin applications of urethan in 3 strains of mice, were able to demonstrate induction or enhancement of pulmonary adenomas, mammary carcinomas, malignant mesenchymal tumours in the interscapular fat, cystoadenomas of the lacrimal gland and blood cysts in the liver. The hemorrhagic cystic lesions in the liver of urethan-treated mice have been observed by several investigators (Kirschbaum, Bell and Gordon, 1949; Roe and Salaman, 1954; Berenblum and Haran, 1955; Heston, Vlahakis and Deringer, 1960; Kawamoto *et al.*, 1961) and differently interpreted. Heston *et al.* (1960) observed an increase in the incidence of hepatomas in urethan-treated C3H mice. Pietra and Shubik (1960) reported the induction of melanotic tumours of the skin of urethan-treated hamsters. For the epidermis of the mouse, urethan has been shown to have an initiating action which can be completed by subsequent or alternate applications of croton oil (Salaman and Roe, 1953; Graffi *et al.*, 1953). In a preliminary report, Lindsay and Bielschowsky (1956) recorded the induction of squamous cell tumours of the skin in mice using topical applications of urethan alone. Urethan given orally induced squamous cell papillomas of the forestomach of the mouse and of the hamster (Berenblum and Haran-Ghera, 1957; Pietra and Shubik, 1960). In the studies of Kawamoto, Kirschbaum and Taylor (1958) and of Berenblum and Trainin (1960), although urethan alone has not been found to exert a leukemogenic action, it has been reported to increase and accelerate the incidence of leukemias induced by X-rays, estrogens or methylcholanthrene. In the most recent report of Kawamoto *et al.* (1961) urethan, given alone, failed to augment the already high incidence of leukemia in AKR and C58 mice and only slightly accelerated its onset. From their study, Berenblum and Trainin (1960) suggested that urethan acts as a promoting factor in leukemogenesis in C57BL/6 mice. However, Pietra, Rappaport and Shubik (1961) observed an early occurrence of lymphomas in Swiss mice injected at birth with urethan.

The present investigation was stimulated by the observation of a few skin papillomas in a group of male Swiss mice given urethan in the drinking water. These papillomas arose on the skin of the lower back where ulcerations and scarring from fighting had occurred. To confirm this finding, 2 groups of mice, males and females, were given urethan in the drinking water. The number of

* Present address: Istituto dei Tumori, Milan, Italy.

skin tumours occurring was again too small to be safely interpreted, but in the same experiment a high incidence of malignant lymphomas was observed.

MATERIAL AND METHODS

Swiss albino mice, from the colony originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and bred randomly in this laboratory since 1951, were used. They were housed in plastic cages with wood shavings in groups of 10 according to sex, fed Rockland diet in pellets and tap water *ad libitum* with the exception to be described.

The experimental group originated from a number of litters born within a few days. At 10 weeks of age, 100 females, weighing from 19 to 26 g. (average 24·8 g.) and 100 males weighing from 24 to 35 g. (average 28·6 g.) were selected and given 0·4 per cent urethan (Fisher Scientific Co.) in the drinking water. After 10 days, the average weight had decreased to 21·7 g. in the females and to 24·8 g. in the males; therefore the treatment was stopped and the animals were given tap water until the original weight was restored. The interval lasted 10 days and was followed by a second, final period of 10 days of urethan treatment, which again resulted in a weight loss and in addition 5 deaths among the males. From the total water consumption during the 20 days of treatment, it was calculated that each female received approximately 240 mg. and each male approximately 320 mg. of urethan.

As a control, 100 females and 100 males, selected in the same way as the experimental animals, were kept untreated.

During the first 40 weeks from the beginning of the experiment, the animals of the experimental group were allowed to die spontaneously or killed when moribund. Then, at the 42nd week, that is at 1 year of age, most of the animals had dyspnea and therefore it was decided to kill all the survivors. The control groups were kept until spontaneous death or killed when in poor condition.

Complete pathological study was done on all animals. In a few instances the histological diagnosis was omitted or limited because of decomposition.

RESULTS

The number of survivors at various ages and the number of malignant lymphomas observed are shown in Table I.

TABLE I.—*The Occurrence of Malignant Lymphomas in Swiss Mice Treated with Urethan and Untreated*

Groups		Number of survivors (weeks of age)										Number of mice with malignant lymphomas (weeks of age)									Total number
		10	20	30	40	50	60	70	80	90	100	15–20	21–25	26–30	31–35	36–40	41–45	46–50	51–60	Over 61	
Urethan 0·4 per cent in drinking water for 20 days	♀	100	98	87	62	30	—	—	—	—	—	0	2	4	13	5	1	1	2	—	28
	♂	100	89	84	56	27	—	—	—	—	—	0	0	1	3	5	0	1	5	—	15
Control	♀	100	100	100	89	82	70	57	41	18	—	0	0	0	0	3	1	1	5	6	16
Control	♂	100	98	89	81	57	45	25	13	—	—	0	0	0	0	0	0	0	2	2	4

In the urethan-treated group, the first 2 lymphomas occurred in females dying at 23 and 24 weeks of age, that is 8 and 9 weeks after the end of treatment. Of 28 lymphomas observed in the females, 24 occurred before the 40th week of age, that is before 25 weeks had elapsed from the end of treatment. The incidence of lymphomas in the urethan-treated males was almost one-half of that in the females ; 9 out of 15 occurred before the 40th week of age.

In the control group, 16 females developed malignant lymphomas ; 10 died before the 60th week of age, only 3 of them before the 40th week. Among the males, fewer lymphomas were observed. Not a single male died of lymphoma before the 40th week of age, 2 died with lymphomas before and 2 others after the 60th week.

The great majority of the lymphomas occurring before the 50th week of age were of lymphocytic type (lymphosarcomas) with different degrees of differentiation. There were also several stem cell lymphomas distributed in both experimental and control groups. Both these types of lymphomas were usually generalized, but on many occasions their preponderant origin in the thymus was still evident. The majority of the lymphomas observed in animals dying after the 50th week were of the histiocytic type (reticulum cell sarcomas). Only 1 urethan-treated female which died at the 66th week had granulocytic leukemia.

Eighty-six females and 78 males of the urethan-treated mice had multiple lung adenomas ; considering that some animals died in the very beginning of the experiment, the incidence of lung adenomas approaches 100 per cent in both sexes. In the control group, 23 females and 9 males died with lung adenomas ; of these 3 of each sex died between the 40th and 60th week, the others after the 60th week. In the urethan-treated group, there were 2 females with mammary tumours which were both recognized at 44 weeks of age. Among the controls, 13 females developed mammary tumours, 6 at 1 year of age, the others after the 60th week. Multiple small hemangiomas of the liver were observed in several urethan-treated animals of both sexes, which died or were killed after the 40th week of age ; the liver parenchyma was otherwise normal. One hemangioma of the uterine wall and 3 hemangiomas of the spleen were also seen. Among the treated animals, 3 males and 2 females had a total of 9 papillomas and 1 sebaceous carcinoma of the skin ; 2 females had multiple papillomas of the forestomach ; 1 male had a renal tubular adenoma.

DISCUSSION

The short-term administration of a large amount of urethan to Swiss mice resulted in an earlier development and higher incidence of malignant lymphomas when compared with untreated Swiss mice of corresponding sex. Females developed twice as many lymphomas as the males. Most of the lymphomas observed before the 50th week were of the lymphocytic type (lymphosarcomas) while beyond that age histiocytic (reticulum cell) sarcomas predominated. Considering that the majority of the lymphomas in the controls occurred after the 50th week, this histological observation seems to corroborate the numerical difference between the experimental and control groups, even though the biological significance of the various types of lymphomas is unknown. However, the sex ratio of the induced lymphomas which followed the ratio observed in the untreated animals of the two sexes, indicates that urethan has really enhanced

and accelerated a process which may have developed without any specific treatment. This situation could be equated with the variation in response to urethan observed in strains which, untreated, have in the same order a different incidence of mammary tumours (Tannenbaum and Silverstone, 1958). There are many similar examples in carcinogenesis and it becomes almost a matter of semantics to decide if each carcinogenic process should be labelled as induction or enhancement.

It is interesting to note that the occurrence of malignant lymphomas or leukaemias with urethan alone has not been reported in the studies of Kawamoto *et al.* (1958) and of Berenblum and Trainin (1960) nor in the long-term experiments of Tannenbaum and Silverstone (1958). In those studies several strains of mice have been used, the lymphoma incidence of which does not appear to be much different from that observed in our untreated Swiss mice. The different result might be explained by the different dosage and way of administration of urethan. The present experiment differs from the others in the larger amount of urethan given continuously for two periods of 10 days each, reaching a transient toxic effect.

The experiments of Berenblum and Trainin (1960) are demonstrative of a promoting action of urethan on leukemogenesis. Although the two periods of treatment of our experiment might be suggestive of some sort of two stage action, it is clear that we do not have data to substantiate this suggestion. For the present, it is only possible to consider that the leukemogenic effect has been achieved by a large amount of urethan given in a short period, which should indicate a direct action of urethan on the lymphatic tissue.

SUMMARY

The administration to Swiss mice of 0·4 per cent urethan in the drinking water for two periods of 10 days with an interval of 10 days, resulted in a transient toxic effect and in the rapid occurrence of malignant lymphomas. Twenty-eight per cent of the females and 15 per cent of the males developed lymphomas, whereas among the controls the incidence was 16 per cent in the females and 4 per cent in the males. In the urethan-treated animals, the majority of lymphomas occurred before the 50th week of age and most of them were of lymphocytic type, while in the controls most lymphomas occurred after the 50th week and were of histiocytic type.

We wish to thank Dr. Henry Rappaport for his help in diagnosing the tumours observed and Mr. Manuel Sueiro for the histological preparations.

This investigation was supported by the U.S. Public Health Service Grant CS-9212.

REFERENCES

Berenblum, I. and Haran, N.—(1955) *Brit. J. Cancer*, **9**, 453.
Idem and Haran-Ghera, N. A.—(1957) *Ibid.*, **11**, 77.
Idem and Trainin, N.—(1960) *Science*, **132**, 40.
Graffi, A., Vlaminck, E., Hoffmann, F. and Schulz, I.—(1953) *Arch. Geschwulst.-forsch.*, **5**, 110.

HESTON, W. E., VLAHAKIS, G. AND DERINGER, M. K.—(1960) *J. nat. Cancer Inst.*, **24**, 425.
KAWAMOTO, S., IDA, N., KIRSCHBAUM, A. AND TAYLOR, H. G.—(1958) *Cancer Res.*, **18**, 725.
Idem, KIRSCHBAUM, A., IBANEZ, M. L., TRENTIN, J. J. AND TAYLOR, H. G.—(1961) *Ibid.*, **21**, 73.
KIRSCHBAUM, A., BELL, E. T. AND GORDON, J.—(1949) *J. Lab. clin. Med.*, **34**, 209.
LINDSAY, D. AND BIELSCHOWSKY, F.—(1956) *Rep. Brit. Emp. Cancer Campgn*, **34**, 372.
PIETRA, G., RAPPAPORT, H. and SHUBIK, P.—(1961) *Cancer*, **14**, 308.
Idem AND SHUBIK, P.—(1960) *J. nat. Cancer Inst.*, **25**, 627.
ROE, F. J. C. AND SALAMAN, M. H.—(1954) *Brit. J. Cancer*, **8**, 666.
SALAMAN, M. H. AND ROE, F. J. C.—(1953) *Ibid.*, **7**, 472.
SHIMKIN, B. M.—(1955) *Advanc. Cancer Res.*, **3**, 223.
TANNENBAUM, A. AND SILVERSTONE, H.—(1958) *Cancer Res.*, **18**, 1225.

GENESIS AND FUNCTION OF MAST CELLS. MAST CELL AND PLASMACYTE REACTION TO INDUCED, HOMOLOGOUS AND HETEROLOGOUS TUMOURS

G. CSABA, T. ÁCS, CECILIA HORVÁTH AND KATALIN MOLD

From the Department of Histology and Embryology, Budapest Medical University, Hungary

Received for publication February 11, 1961

It was observed in earlier experiments of the present authors that the introduction of heparin components into the organism (i.e. glucuronic acid and glucosamine) promoted, while the chemical binding of heparin inhibited, the growth of experimental tumours. Combined treatment with toluidine blue, thionine or protamine sulphate, i.e. with heparin-binding substances, resulted in a marked inhibition of malignant growths in test animals (Csaba, Horváth and Ács, 1960; Csaba, Ács, Horváth and Kapa, 1960). It has long been known that there exists a certain correlation between tumours and polysaccharides (Almquist and Lansing, 1957; Asboe-Hansen, 1954; Koenig, 1955; Rottimer, Levy and Conte, 1958; Weimer, Quinn, Redlich-Moshin and Nishihara, 1957; Winsler and Smyth, 1948); certain authors actually regard heparin as a growth-inhibitor (Balázs and Holmgren, 1949; Koenig, 1955). Though opinions are fairly contradictory, authors are in agreement regarding the observation that a multiplication of mast cells invariably occurs in the vicinity of tumours, and that both the appearance and multiplication of these heparin-containing cells are associated with progressive tumorous growth (Fromme, 1906; Higuchi, 1930; Quensel, 1933; Staemmler, 1921; Weill, 1919).

Our earlier investigations into the origin of mast cells and their capacity to take up heparin convinced us that the thymus played a prominent part in the production of mast cells. They arise in this organ from reticular cells as also from large and medium-sized thymocytes. A reaction of the thymic tissue can be observed in tumorous organisms or those in a state of tissue proliferation: cysts containing a substance sensitive to Schiff's periodic reaction (PAS-positive) are formed in the thymus, and this process is associated with the appearance of PAS-positive thymocytes, the major part of which changes into mast cells (Csaba and Kapa, 1960; Csaba, Törö, Ács and Kiss, 1960; Csaba, Törö and Kapa, 1960). These investigations seemed to prove that the presence of tumours meant a provocative stimulus for the thymus, while the question remained open whether the mast cells appearing in the vicinity of the tumours were, or were not, of thymic origin. Such origin appears to be the more questionable as the entire polysaccharide metabolism of tumorous organisms undergoes modifications which must affect the mast cells as well.

We tried to approach both these problems in our experiments: (a) we studied mast cell reaction to tumours of different origins (homologous and heterologous transplantations affording, at the same time, a possibility of studying local

immune reactions also); (b) we endeavoured to collect data about the origin of mast cells. Such arrangement of the experiments enabled us to observe tissue reaction in a state of disturbed tissue correlation (induced tumour), in a state of tissue immunity (heterologous transplantation) and in cases where disturbed harmony of the tissues was associated with tissue immunity (homologous transplanation).

METHOD

A total of 160 albino mice, obtained from the stock of the National Institute of Public Hygiene, were used as test animals. We divided them into three groups. Group I consisted of 60 mice: after depilating their skin, we painted it with a 0·5 per cent concentration of benzopyrene dissolved in benzene, every other day during a month and at intervals of 4 or 5 days thereafter. Members of Group II (60 mice) were intracutaneously injected with 0·01 to 0·02 ml. of Ehrlich ascites tumour, while those of Group III (40 mice) received, likewise intracutaneously, 0·01 to 0·02 ml. of Yoshida tumour suspension.

We removed the first test materials 24 hours after the first treatment and inoculation, respectively; test samples were taken hereafter every day during 10 days, and at intervals of 4 to 5 days afterwards. What we isolated was, first, the painted area of the skin or the site of the intracutaneous inoculation, and, thereafter, the entire tumour which had developed, together with the skin covering it. We made, moreover, preparations of the membrane obtained from the subcutaneous connective tissue situated beneath the painted area of the tumour.

The material was fixed in Carnoy's fluid; we made sections at four levels from each sample, and treated them (as also the membranes) with Giemsa's stain, toluidine blue or methylgreen pyronin. The PAS reaction, too, was performed.

RESULTS

Disturbance of tissue correlation was represented in our experiments by induced tumours, homologous transplants by Ehrlich's tumour, and heterologous transplants by Yoshida's tumour.

Time of observations:

1. Induced tumour: samples were taken and examined 19 times, the last at the appearance of cornification, or, microscopically, of the first tumour nest.

2. Ehrlich: removal and examination were performed 19 times, the last at the time when massive ulceration of the skin had begun.

3. Yoshida: removal and examination 9 times, the last at the time when the tumour had ceased to be palpable.

Tumours induced by benzopyrene (Fig. 1 and 2)

Twenty-four hours after first painting: No conspicuous change observable either in the epithelium or the connective tissue.

Forty-eight hours: Considerably increased number of mast cells, mostly appearing in the connective tissue, at some distance from the epithelium, between the sebaceous glands or the hair follicles. They are intensively granulated, of various shapes, and only a few of them show the regular form of mast cells. One sees mostly cells with processes which resemble either fibroblasts or macrophages.

Seven days: Number of mast cells as after 48 hours. Advancing cornification of epithelium; pycnosis and karyorrhexis observable in the cells. The phenomenon becomes pronounced on the 5th day when the epithelium forms cones in the cutis and also the epithelium of the hair follicles begins to proliferate. These follicles show dilatation so that the hairs they contain appear as pearls; epithelial degeneration and much nuclear fragmentation can be seen. Metachromatic matter accumulates around the swollen hair follicles, and it is not possible to determine at this time whether this substance derives from the broken-up mast cells.

Ten days: Further progress of cornification, with the appearance of vast numbers of mast cells. Many irritomotile figures among the other connective tissue cells. Mast cells provided with numerous processes, their granulation uneven. They are hyperchromatic, but number of disintegrated cells is still very low.

Fifteen days: Further increase in the number of mast cells. These, too, are provided with processes and stain metachromatically. They are situated in the cutis, some of them near the epithelium. Many of the mast cells are broken up and their granules scattered in the connective tissue.

Twenty-three days: The epithelial structure appears to be basally loosened at certain points where a direct contact between mast cells and epithelial cells is established.

Samples taken on the 28th, 33rd, 39th, 44th, 50th and 57th days show a high degree of cornification; one can well observe the penetration of epithelial cones towards the deeper layers and, as from the 40th day, the disintegration of epithelial structure. Contact between mast cells and epithelial cells is so close by the 33rd day that occasional mast cells appear among the epithelial cells both in the epithelial cones and the hair follicles. They show the typical form of mast cells and are so numerous in the immediate vicinity of the epithelium that the epithelial cells are fully covered up at certain points. Cells, provided with nuclei characteristic of epithelial cells, can be seen in close contact with the epithelial cones, as from the 40th day. Vast numbers of mast cells are degenerating in certain areas. Hardly anything but mast cells are visible in certain parts of the connective tissue, especially in the neighbourhood of the epithelium.

Sixty-two days: Associated with one of the epithelial cones, the appearance of tumorous substance can be observed. Mast cell reaction is very intensive around the tumour cells.

PAS reaction revealed no pronounced increase in the amount of neutral mucopolysaccharides. While no plasmacytes were observable in the preparations stained with methylgreen pyronin, they showed the presence of numerous mast cells which were vividly metachromatic upon being stained with methylgreen.

Only a moderate number of regularly shaped mast cells was observable in the membrane preparations on the 2nd day. They became, however, exceedingly numerous after 10 days, and it was at this time that their disintegration began. The cells contained many vacuoles, and their hypergranulation increased as time went on. There were numerous disintegrated figures also among the mast cells situated beside the capillaries. Disintegration was so rapid that only occasional unimpaired mast cells had remained by the 39th day. The entire subcutaneous connective tissue had filled up with granules in the area of painting. Even the remaining few more or less unimpaired mast cells were stuffed with granules waiting to be scattered.

Ehrlich's tumour-homologous transplantation (Fig. 3 to 12)

At 24 hours after transplantation : The implanted cells, forming densely packed bundles, are situated in the cutis. Detritus is observable in certain areas. That the cells are alive is shown by the intensive pyroninophilia of the cytoplasm.

Forty-eight hours : The central portion of the implant becomes necrotic, with the marginal part remaining alive. A few mast cells observable towards the epithelium, among the sebaceous glands. Tumour cells begin to spread between the connective tissue cells.

Four days : The necrotic part is almost completely absorbed in some, and still present in other, cases. Migration of the cells is pronounced : the emigrant cells form epithelial islets in which many atypical mitoses can be seen. Mast cell reaction becomes stronger.

Five days : Tumorous growth is more advanced. It is on the 5th day that a contact between mast cells and tumour cells is established on that aspect of the tumour which is directed towards the epithelium. Tumour cells approximate, sometimes even reach, the epithelium.

Six days : Mast cell reaction becomes more and more pronounced : the visual field of a 10×-objective shows 60 to 70 mast cells in the connective tissue. They have either a regular shape or show a number of processes. Even cells containing metachromatic granules are sometimes observable between the connective tissue cells, always near the epithelium.

Seven days : Further increase in the number of mast cells. They begin to disintegrate in certain areas. Recurrence of central necrosis as the tumour is spreading. Mast cells already visible between tumour cells.

EXPLANATION OF PLATES

FIG. 1.—Skin treated with benzopyrene ; preparation of membrane from subcutaneous connective tissue on 2nd day. Toluidine blue. ×100.

FIG. 2.—Skin treated with benzopyrene ; preparation of membrane from subcutaneous connective tissue on 12th day. Toluidine blue. ×200.

FIG. 3.—Intracutaneously implanted Ehrlich tumour ; 6th day. Appearance of mast cells beneath epithelium, far from tumour. Two mast cells of different types visible in enframed area. Toluidine blue. ×100.

FIG. 4.—Enlarged detail of Fig. 3. The letter E indicates cell of the epithelial, the letter C that of the connective-tissue type. Toluidine blue. ×400.

FIG. 5.—Intracutaneously implanted Ehrlich tumour ; 6th day. Numerous mast cells adjacent to epithelium. Giemsa stain. ×100.

FIG. 6.—Intracutaneously implanted Ehrlich tumour ; 12th day. Mast cells detaching from epithelium. Toluidine blue. ×400.

FIG. 7.—Intractuaneously implanted Ehrlich tumour ; 12th day. Mast cells detaching from epithelium (indicated by M). Giemsa stain. ×400.

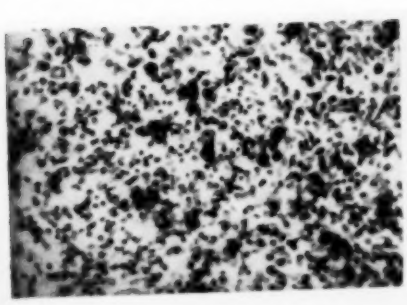
FIG. 8.—Intracutaneously implanted Ehrlich tumour ; 12th day. Mast cells detaching from epithelium (indicated by M). Giemsa stain. ×400.

FIG. 9.—Intracutaneously implanted Ehrlich tumour ; 18th day. Vast numbers of mast cells in immediate contact with epithelial elements. Toluidine blue. ×100.

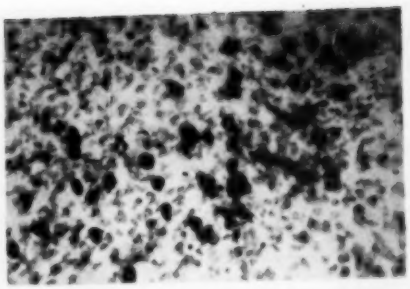
FIG. 10.—Intracutaneously implanted Ehrlich tumour ; 18th day. Mast cells in close contact with epithelium. Toluidine blue. ×100.

FIG. 11.—Intracutaneously implanted Ehrlich tumour ; 21st day. Epithelium approached and destroyed by tumour. Mast cells of epithelial character (indicated by M). Toluidine blue. ×200.

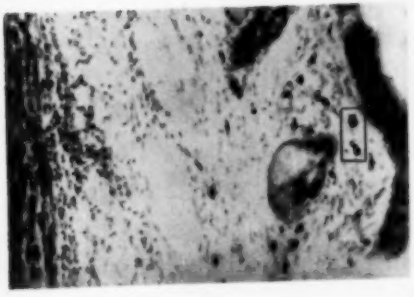
FIG. 12.—Subcutaneous connective tissue 38 days after implantation of Ehrlich tumour. Numerous disintegrating or disintegrated mast cells. Membrane preparation. Toluidine blue. ×200.



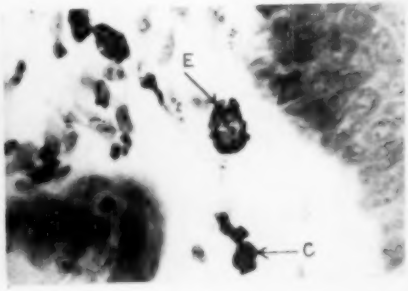
1



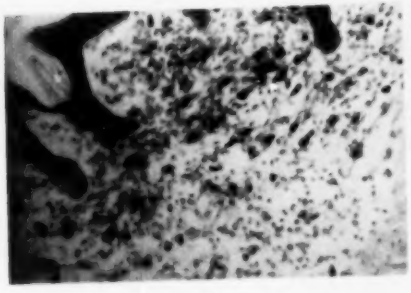
2



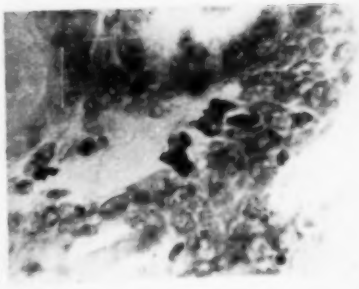
3



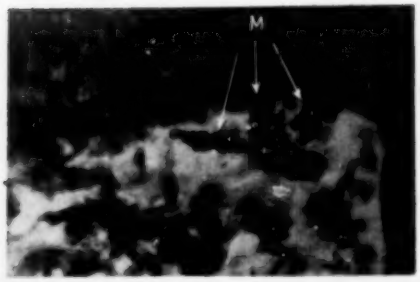
4



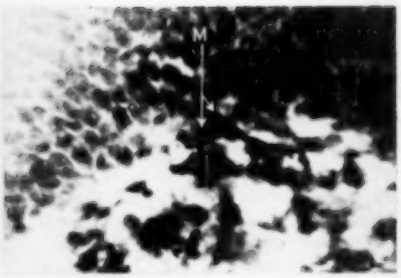
5



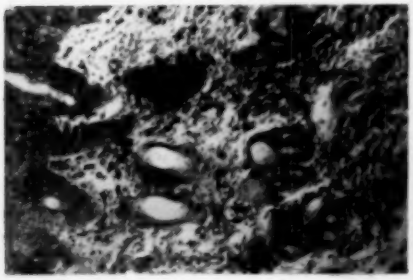
6



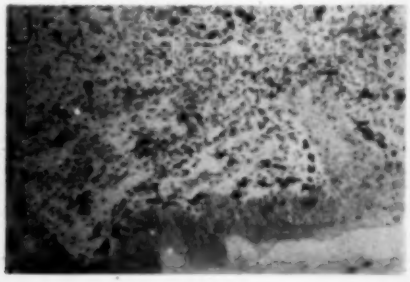
7



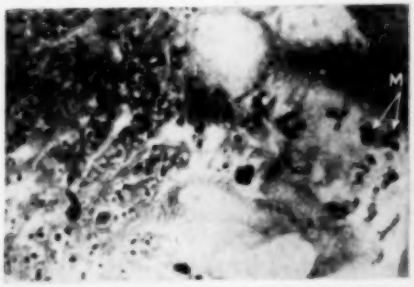
8



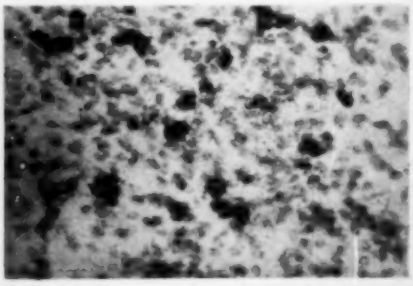
9



10



11



12

Eight to twelve days : Further growth of tumour and progress of mast cell multiplication.

Twelve days : Further disintegration of mast cells, vast numbers of which are to be found in the tumour and around the epithelial cells. Certain of the latter fill up with metachromatic granules and become detached. Whole picture dominated by mast cell transformation. Tumour reaches epithelium at some points, giving rise to ulceration.

Eighteen days : Further increase in the number of mast cells. Those situated near the epithelium are paler, less granular and show weak azure metachromasia ; those lying near the tumour are hypergranulated, show a darker colour and tend to disintegrate.

Twenty-one days : Number of mast cells less than before, since tumour has reached the epithelium almost everywhere, but transformation of epithelial cells into mast cells still observable. Cells detach themselves from the Malpighian layer, and it can be well seen that the nuclear structure of intra-epithelial mast cells is the same as that of cells situated in the germinative layer.

Twenty-four to thirty-eight days : Tumour attains the size of a hazelnut ; general ulceration ; occurrence of mast cells at points where contact between epithelium and tumour not yet established, but most of them situated in the tumorous substance even at such points.

Great numbers of strongly garnulated mast cells, arranged in groups, can be seen to appear in the membrane preparations, i.e. in the subcutaneous layer, 24 hours after transplantation. Their further growth and proliferation take practically the same course as that described in connection with induced tumours. Staining with methylgreen pyronin revealed no plasmacytes either in the membranes or the sections.

Yoshida's tumour-heterologous transplantation

Much detritus and many degenerated cells can be seen as early as 24 hours after the inoculation, and practically all cells become necrotic after 48 hours. Both connective tissue reaction and plasmacyte reaction begin on the 4th and become very pronounced on the 7th day. The tumour ceases to be palpable at the site of inoculation on the 8th day, its place being taken everywhere by connective tissue.

Plasmacyte reaction begins on the 4th to 5th day, and takes its full course until about the 8th day. Mast cell reaction appears on the 7th day and is still in progress on the 8th and 9th day. Mast cells resemble either medium sized thymocytes or macrophages.

Some of the plasma cells are true to form, but there occur also cells which, though plasmacyte-like, have no typical nuclei, while their cytoplasm is strongly pyroninophilic. They occur mostly in groups with only a few being scattered.

The greatest number of mast cells are to be found between the proliferating connective tissue cells.

DISCUSSION

We observed the most pronounced mast cell reaction in cases of induced tumour ; it was almost as intensive in cases of homologous transplantation (Ehrlich's tumour), while a decidedly weak reaction followed heterologous trans-

plantations (Yoshida's tumour). On the other hand, plasma cell reaction occurred only in cases of heterotransplantation.

What are we justified to conclude from these observations ?

It emerged from our earlier experiments that mast cell reaction was the organism's response to disturbances affecting the correlation of tissues, e.g. to tumorous growths (Csaba, Törö and Kiss, 1959). A reaction of this kind was observed in the thymus. These earlier observations seem to be substantiated by the results of our present experiments : derangement of the harmony between tissues, i.e. tissue proliferation, occasioned the appearance or formation of an exorbitantly large number of mast cells, a phenomenon quite in harmony with the observations of Ehrlich (1877), Westphal (1880), Higuchi (1930), Asboe-Hansen (1954), Asboe-Hansen, Levi and Wegelius (1957) and Lengyel and Vértes (1953) who reported on the appearance of numerous mast cells in the vicinity of tumours. However, the experiments of these authors failed to clear up the cause of this phenomenon. Our present experiments seem to justify us in suggesting that it is not the simple presence of tumour but the process of proliferation which mobilizes mast cells : we have seen that Yoshida's tumour, which belongs to the non-proliferative type, provoked but a very mild mast cell reaction, and even that at a time when the absorption of the growth, the process of repair, had already begun, i.e. coincidentally with connective tissue proliferation. It shows that mast cell reaction was associated also in this case with tissue motility rather than with the presence of tumour, while the influence of foreign proteins gave rise to the appearance of plasma cells, so that the picture was dominated by the representatives of tissue immunity.

It has been noted that only a part of the plasma cells showed the " classic " form of plasmacytes ; yet, although the spoke-like arrangement of the nuclear structure was not observable in others, other morphological features and their strongly pyroninophilic cytoplasm revealed also these forms as plasmacytes. Mast cells showed still wider variations of form. A " regular " mast cell has, as is known, a round or oval shape and contains in its cytoplasm granules which react with metachromasia to toluidine blue or azure ; its nucleus, staining bright with these dyes, is situated in the centre or slightly eccentrically. Although there occurred a few which showed this " classic " form, most of the mast cells in the neighbourhood of tumours displayed significantly different morphological features. The greatest number—especially those situated in the loose connective tissue—had processes and contained atypical nuclei ; the granularity of their cytoplasm was not always pronounced : instead of granules, a homogeneous metachromatic substance was observable in certain instances, while—in other cases—the mast cells rather resembled fibroblasts, macrophages or epithelial cells.

A survey of literature on the origin of mast cells reveals the fact that mast cells may arise from a great number of other cell types. While macrophages are transformed into mast cells according to Velican and Velican (1959) it is suggested by Burkl (1952) that mast cells arise as a result of the storage of heparin by histiocytes ; again, other authors derive them from fibroblasts, while our investigations seem to show that most of the mast cells originate in the thymus from the reticular cells of the epithelium or the large and medium-sized thymocytes. If one accepts all these suggestions (and our present experiments make us inclined to do so) we have to regard as mast cells all cells containing metachromatic granules in the cytoplasm which are scattered when their number exceeds a certain limit. We

think this definition covers all afore-mentioned forms. We do not want to suggest that all mast cells are of equal value, and this the less so as a notion of this kind takes but a single component of the mast cell into consideration, namely heparin, although it contains also histamine, serotonin and a number of other substances.

It appears from our present experiments that the first mast cells appearing around tumours arise from the connective tissue and subsequent ones from the epithelium, while those present in the subcutaneous connective tissue are rather suggestive of the " classic " form of mast cells, i.e. of thymocytic origin. Mast cells of epithelial origin require, however, further discussion. They appear in an advanced phase of tumorous proliferation in the case of both Ehrlich's tumour and in that of protracted cancerogenic painting. Yet, also those mast cells which we regard as originating from connective tissue cells emerge close to the epithelium, and appear in larger numbers near the epithelium even when the tumour is still at a distance from it. That this is so has been confirmed in the course of our earlier experiments conducted in connection with the thymus. It was found that induction by the epithelium was required for the emergence of mast cells and that the epithelial cells themselves changed into mast cells at a certain stage. That the impulse given by the epithelium is of high significance is well borne out by literature according to which mast cell reaction is much weaker in cases of sarcoma than in those of carcinoma, so that, apart from the process of proliferation, the epithelial or non-epithelial character of tumours, too, influences the intensity of mast cell reaction.

The fact that, with advancing proliferation, part of the epithelium itself changes into mast cells, seems to indicate the defensive character of the reaction, as has been recognized by a number of earlier authors. It was found, for instance, by Cramer and Simpson (1944) that mast cell reaction was more marked in animals that showed stronger resistance to the growth of induced tumours. Koenig (1955) claims that mitosis of tumour cells comes to a standstill if great numbers of mast cells are present. That the presence of mast cells inhibits tumorous growth is attributed by these authors to heparin which is liberated by disintegrating mast cells : this substance is known as an antagonist of hyaluronidase and an inhibitor of mitoses (Glick and Sylvén, 1951 ; Greenstein, 1954 ; Holmgren and Wohlfahrt, 1949 ; Balázs and Holmgren, 1949 ; Harding, 1949 ; Fischer, 1936 ; Heilbrunn, 1956 ; Heilbrunn and Wilson, 1949). Our earlier investigations do not support this view : heparin (or rather, its components) were found to promote malignant growth, and tumours—far from being antagonized by heparin—seemed to be in need of this agent for their development. This is substantiated by the findings of Panizzari and Vegeto (1958), as also by those of Ozzello, Lasfargues and Murray (1960). In the light of our own observations and those of the last-named authors, it would appear that mast cells antagonize tumorous growth by taking up, and so depriving tumour cells of, heparin : mast cells appear as a kind of competitor of tumour cells in their quest of heparin, so that the mechanism of inhibition would seem to operate not through a release of heparin but its cellular neutralization. The defence of the organism would, therefore, consist in heparin being taken up by the cells of the connective tissue and the epithelium, their consequent transformation into mast cells, and not the other way round. That this hypothesis is correct seems to be corroborated by the observation that mast cells in the vicinity of the epithelium contain less heparin than those near the tumour (Sylvén, 1945, came to the same conclusion) which shows that cells take up heparin " en route ",

arrive at the tumour in the form of mature mast cells and release their heparin content in its vicinity. Mast cells, therefore, do not produce heparin but absorb and neutralize this substance.

As regards disintegration of mast cells, this occurs frequently both near the tumour and in the more distant subcutis with advancing proliferation. This would mean, as has already been pointed out by us, that the decomposition of mast cells is not due to mechanical traumatization but to a factor which becomes operative in the organism with the spread of tumorous growth. It is possible that the defence mechanism of the organism is overthrown by the tumour which utilizes the heparin of mast cells in such cases for its own growth, but it is likewise possible that the tumour is unable to utilize it in that complex form in which heparin is contained in mast cells. Nor is it impossible that, at this time, the hyaluronidase antagonism of whole heparin is already stronger than the tumour promoting effect of the individual heparin components. No doubt, there seems to exist a certain contradiction between defence by means of mast cells and the fact that these cells so-to-say " dish up " their heparin content to the tumour ; the problem needs further investigation before we can expect to solve it.

To sum up : mast cell reaction seems to be a characteristic concomitant of states in which tissue correlation is disarranged, since it occurs also in pregnancy, wound healing and processes of regeneration, while plasmacyte reaction appears as the local manifestation of tissue immunity. It is quite possible that both reactions are but different cytological manifestations of the organism's defence. Our experiments failed to clear up the question why only mast cell reaction (i.e. one characteristic of states of correlation disturbances) appeared in cases of homologous tumour transplantation, although the presence of homologous protein would have justified the appearance of plasma cell reaction. It seems that, when the transplant (namely Yoshida's tumour) was weakly proliferative but strongly antigenic, antigenicity prevailed and gave rise to plasma cell reaction, while, when the transplant (namely Ehrlich's tumour) was weakly antigenic but strongly proliferative, proliferation prevailed and gave rise to mast cell reaction.

SUMMARY

Experiments are described, made with a view to studying the origin and function of mast cells in the vicinity of induced (benzopyrene), homologous (Ehrlich) and heterologous (Yoshida) tumours. It was found that the organism responded with mast cell reaction to disturbances of tissue correlation, and developed plasma cell reaction in states of tissue immunity. It is suggested that mast cells, which may have various origins, inhibit tumorous growth by neutralizing the polysaccharides, necessary for a development of tumours, in the course of their genesis, i.e. during the process in which other cell types are transformed into mast cells. Various theories regarding the origin of mast cells and their tumour inhibiting function are discussed.

REFERENCES

ALMQUIST, P. O. AND LANSING, E.—(1957) *Scand. J. clin. Lab. Invest.*, **9**, 179.
ASBOE-HANSEN, G.—(1954) " The Mast Cell " in ' International Review of Cytology ', ed. Bourne and Danieli. New York (Academic Press), Vol. 3.
Idem, LEVI, H. AND WEGELIUS, O.—(1957) *Cancer Res.*, **17**, 792.

Balázs, A. and Holmgren, H.—(1949) *Proc. Soc. exp. Biol. N.Y.*, **2**, 142.
Burkl, W.—(1952) *Wien. klin. Wschr.*, **64**, 411.
Cramer, W. and Simpson, W. L.—(1944) *Cancer Res.*, **4**, 601.
Csaba, G., Ács, T., Horváth, G. and Kapa, E.—(1960) *Brit. J. Cancer*, **14**, 367.
Idem. Horváth, C. and Ács, T.—(1960) *Ibid.*, **14**, 362.
Idem and Kapa, E.—(1960) *Nature, Lond.*, **187**, 711.
Idem, Törö, I., Ács, T. and Kiss, F. I.—(1960) *Acta morph. hung.*, **9**, 187.
Idem, Törö, I. and Kapa, E.—(1960) *Ibid.*, **9**, 197, 291.
Idem, Törö, I. and Kiss, F. I.—(1959) *Orientacion Medica*, **8**, 1.
Ehrlich, P.—(1877) *Arch. mikr. Anat.*, **13**, 263.
Fischer, A.—(1936) *Protoplasma*, **26**, 377.
Fromme, E.—(1906) *Zbl. Gynäk.*, **30**, 1146.
Glick, D. and Sylvén, B.—(1951) *Science*, **113**, 388.
Greenstein, J. P.—(1954) 'Biochemistry of Cancer'. New York (Academic Press).
Harding, D.—(1949 *Proc. Soc. exp. Biol. N.Y.*, **71**, 14.
Heilbrunn, L. V.—(1956) 'The Dynamics of Living Protoplasma.' New York (Academic Press).
Idem and Wilson, W. L.—(1949) *Proc. Soc. exp. Biol. N.Y.*, **70**, 179.
Higuchi, K.—(1930) *Folia haemat., Lpz.*, **41**, 401.
Holmgren, H. and Wohlfahrt, G.—(1949) *Cancer Res.*, **7**, 686.
Koenig, H.—(1955) *Z. exp. Med., Moskau*, **126**, 67.
Lengyel, J. and Vértes, B.—(1953) *Orv. Hétil.*, **94**, 657.
Ozzello, L., Lasfargues, E. Y. and Murray, M. R.—(1960) *Cancer Res.*, **20**, 600.
Panizzari, G. D. and Vegeto, A.—(1958) *Arch. ital. Pat. clin. Tumori*, **2**, 1219.
Quensel, V.—(1933) *Acta path. microbiol. scand.*, Suppl. 102.
Rottimer, A., Levy, A. L. and Conte, A.—(1958) *Cancer*, **11**, 351.
Staemmler, M.—(1921) *Frankfurt. Z. Path.*, **25**, 391.
Sylvén, B.—(1945) *Acta Radiol.*, Suppl. 59.
Velican, C. and Velican, D.—(1959) *Acta morph. hung.*, **9**, 11.
Weill, P.—(1919) *Folia haemat., Lpz.*, **23**, 185.
Weimer, H. E., Quinn, F. A., Redlich-Moshin, J. and Nishihara, H.—(1957) *J. nat. Cancer Inst.*, **19**, 409.
Westphal, E.—(1880) Dissertation, Berlin.
Winsler, R. J. and Smyth, J. M.—(1948) *J. clin. Invest.*, **27**, 617.

THE SURVIVAL TIME OF MICE WITH EHRLICH'S ASCITES CARCINOMA RELATED TO THE SEX AND WEIGHT OF THE MOUSE, AND THE BLOOD CONTENT OF THE TUMOUR

F. HARTVEIT

From the University of Bergen, School of Medicine, the Gade Institute, Department of Pathology, Bergen, Norway

Received for publication April 20, 1961

It appears to be generally accepted that Ehrlich's ascites carcinoma will grow in all mice (Karnofsky, 1953), with no spontaneous remissions (Lettré, 1941). Klein and Révész (1953) showed that a minimum of 400,000 tumour cells was necessary to obtain ascitic growth in all cases. However, although the death of the mouse is certain within a time limit dependent on the tumour cell dose, the scatter in the survival time has not been investigated.

The tumour grows equally well in male and female mice, although Ahlström and Ising (1955) have shown that there is a difference in its growth in male and female hamsters. The tumour cells and the inflammatory cells present in the tumour ascites have been investigated at the expected median survival time of the mice (Klein, 1950), but no reference has been found to any investigation into the amount of blood present in this ascites, or its significance.

This experiment was therefore designed to see if the sex and weight of the young adult mice kept at this Institute had any bearing on their survival time after the intraperitoneal injection of Ehrlich's ascites carcinoma, and further to see if there were any difference in the amount of blood in the tumours of mice dying spontaneously at different times after injection.

MATERIAL AND METHODS

Twenty-five male and 25 female mice were used. All were adult but under 6 months old. They were taken from a closed colony of previously inbred white mice obtained from Professor Kreyberg in Oslo.

The Ehrlich's ascites carcinoma was originally obtained from Professor Ahlström in Lund who had earlier got it from Klein in Stockholm. At the time of the experiment the tumour was in its 67th transplant generation here.

All the mice were weighed and marked. Each mouse was then given one intraperitoneal injection of 0·1 ml. tumour ascites (a tumour cell dose of 1,860,000/mm.c.) taken from one mouse. This mouse had been injected with Ehrlich's ascites carcinoma 10 days before.

The mice were kept in cages of 5. When a mouse died the survival time was recorded in days. Then the abdomen was opened and the tumour ascites removed and measured. One ml. of the fluid was centrifuged at 2,400 r.p.m. for 45 minutes. The volume of the red blood cells that formed a dark layer at the bottom of the Wintrobe tube was taken as an estimate of the amount of blood present per ml., and recorded as a percentage of the total volume in the tube.

RESULTS

Fig. 1 shows the distribution of deaths related to time. It will be noticed that this curve has two peaks, marked a and b. If the mean survival time of the total series, 10·5 days, is taken as the dividing point, the mean survival time is 7 days for group a and 13·03 days for group b.

Table I shows the mean survival time and its standard deviation (SD) for the mice (a) dying before the mean survival time for the total series (corresponding to the first peak in Fig. 1) compared to that of the mice (*b*) dying after this mean survival time (corresponding to the second peak). The table also gives the mean

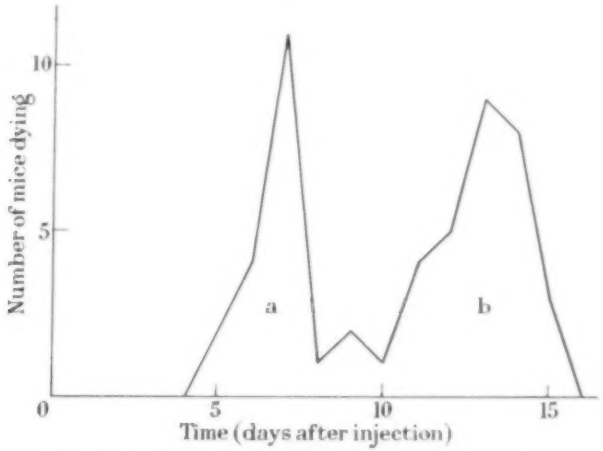


FIG. 1.—The distribution of the deaths of 50 mice dying after the intraperitoneal injection of Ehrlich's ascites carcinoma.
a. Peak corresponding to group a.
b. Peak corresponding to group b.

amount of blood/ml. tumour ascites and the final volume of the tumour ascites, with the SD of these means for groups a and b. The standard error (SE) of the actual difference between the means of the groups, the *t* and *P* values are shown for all three factors. These findings indicate that the difference between both

TABLE I.—*The Survival Time of the Mice in Groups a and b, and the Blood Content and Final Volume of the Tumours, Showing the Number of Mice, the Mean Values with SD, the SE of the Difference Between the Group Means, the* t *and* P *Values.*

x	Group	*n*	$\bar{x}$	SD$\bar{x}$	SE$\bar{x}_a - \bar{x}_b$	*t*	*P*
Survival time (days)	a	21	7·0	1·196	0·346	17·42	0·001 > *P*
	b	29	13·03	1·225			
Blood vol./ml. ascites (%)	a	21	12·23	4·096	1·125	9·14	0·001 > *P*
	b	29	1·91	1·114			
Volume of ascites (ml.)	a	21	2·74	1·146	1·770	2·564	0·02 > *P* > 0·01
	b	29	7·28	2·983			

the survival time and the amount of blood present in the tumour in both groups is very highly significant statistically, while the difference in the final volume of the tumour ascites is also significant.

Fig. 2 shows the regression lines for the scatter diagrams when (1) the volume of tumour ascites is related to the survival time ($y = 51{\cdot}68 - 2{\cdot}869x$), and (2) the blood volume/ml. tumour ascites is related to the survival time ($y = 22{\cdot}17 - 1{\cdot}54x$). From this figure it is evident that the amount of tumour present in the dead mice is greater in those surviving longer, while the amount of blood in the tumour is greater in the mice dying after a short survival time.

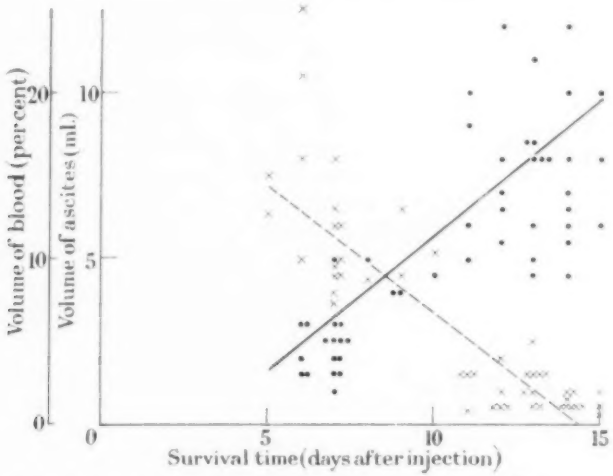


FIG. 2.—Scatter diagram and regression line for :—
1. Volume of tumour ascites (●) related to survival time (———).
2. Blood volume/ml. ascites (×) related to survival time (– – – –).

Table II gives the mean values for the starting weight, survival time, volume of tumour ascites, blood volume/ml. ascites and total blood volume in the ascites for the total series (♂ + ♀) and for the males and females alone. In addition to the number of animals involved, the mean values with SD, the SE of the differences between the male and female means, the t and P values are shown.

From this table it is evident that although the difference in the mean starting weight for the male and female series is highly significant statistically, the differences in the mean survival time, volume of tumour ascites, volume of blood/ml. tumour ascites and total blood volume in the tumour ascites are not significant.

Table III shows the correlation between the starting weight and the final volume of tumour ascites, and between the survival time and the following factors : the starting weight, the volume of tumour ascites, the volume of blood/ml. ascites and the total blood volume in the ascites. The table gives the number of animals used, the correlation coefficient (r), the t and P values for the various factors.

It can be seen from Table III that the correlation between the starting weight and the final volume of tumour ascites is not statistically significant in the male

TABLE II.—*The Distribution of the Factor* x *for the Total, the Male and the Female Series, Giving the Number of Mice, the Mean Values with SD, the SE of the Difference between the Male and Female means, the* t *and* P *Values.*

x	Series	n	$\bar{x}$	$SD_{\bar{x}}$	$SE_{\bar{x}_\delta - \bar{x}_\varphi}$	t	P
Starting weight (g.)	♂+♀	50	18·58	4·858			
	♂	25	20·52	1·691	1·374	2·833	0·01>P>0·001
	♀	25	16·64	6·075			
Survival times (days)	♂+♀	50	10·5	3·208			
	♂	25	11·0	3·349	0·9203	1·087	0·1>P>0·05
	♀	25	10·0	3·222			
Volume of ascites (ml.)	♂+♀	50	5·57	2·292			
	♂	25	6·14	3·211	0·8258	1·380	0·2>P>0·1
	♀	25	5·0	2·466			
Blood/ml. ascites (%)	♂+♀	50	6·0	5·771			
	♂	25	5·98	6·059	1·635	0·1761	0·9>P>0·8
	♀	25	6·02	6·020			
Total blood (ml. × %)	♂+♀	50	21·54	13·07			
	♂	25	21·17	12·71	3·696	0·5195	0·7>P>0·6
	♀	25	23·09	11·25			

series. However, for the female and the total series this correlation is significant at the 5 per cent level. The correlation between the survival time and the starting weight is not significant in any of the series. There is a highly significant positive correlation between the survival time and the volume of tumour ascites, and a highly significant negative correlation between both the survival time and the blood/ml. ascites and the total blood in the ascites for all the series.

TABLE III.—*Correlation Between Factors* x *and* y, *Showing the Number of Animals Used, the Correlation Coefficient* (r), *the* t *and* P *Values, for the Total, the Male and the Female Series.*

x	y	Series	n	r	t	P
Starting weight (g.)	Volume of ascites (ml.)	♂+♀	50	0·3226	2·258	0·05>P>0·02
		♂	25	−0·1733	0·8492	0·4>P>0·3
		♀	25	0·5077	2·488	0·05>P>0·02
Survival time (days)	Starting weight (g.)	♂+♀	50	0·07679	0·537	0·6>P>0·5
		♂	25	−0·1547	0·7582	0·5>P>0·4
		♀	25	0·2134	1·046	0·4>P>0·3
Survival time (days)	Volume of ascites (ml.)	♂+♀	50	0·7998	5·593	0·001>P
		♂	25	0·7632	3·74	
		♀	25	0·8472	4·152	
Survival time (days)	Blood/ml. ascites (%)	♂+♀	50	−0·8506	5·94	0·001>P
		♂	25	−0·9166	4·49	
		♀	25	−0·7539	3·694	0·01>P>0·001
Survival time (days)	Total blood (% × ml.)	♂+♀	50	−0·6992	4·89	0·001>P
		♂	25	−0·7338	2·596	0·01>P>0·001
		♀	25	−0·6750	3·308	

DISCUSSION

Although the mice used in this experiment were all taken from the same closed colony and each received the same dose of Ehrlich's ascites carcinoma cells from the same mouse, some scatter in their survival time was expected. The two-peaked curve that was obtained when the number of deaths was related to the time after injection suggests, however, that the mice dying of this tumour fall into two distinct groups, as is evident from Fig. 1. The SD in both groups is low and the means are separated by as much as 6·03 days. The chance that both these groups were taken from the same population is less than 1 : 1000. Thus the mice used here react in two ways to the same treatment.

This experiment shows that neither the sex nor the weight of the mouse influenced the survival time. There was, however, a highly significant positive correlation between the survival time and the final volume of the tumour, showing that the tumour grows progressively. This is in accordance with Klein's (1950) findings. There was also a positive correlation between the final volume of tumour and the starting weight of the mouse. This correlation is significant at the 5 per cent level in the total and female series, but not in the male series. This is probably accounted for by the greater scatter in the weights of the female mice and by the fact that the female mice were, on the whole, smaller than the male. This shows that the smaller mice produced smaller amounts of tumour.

When the ascitic variant of the Ehrlich mouse carcinoma was first obtained the formation of " a huge ascites of milky or bloody character " was described occurring 10–14 days after intraperitoneal inoculation (Loewenthal and Jahn, 1932). Klein (1950) quotes this statement but makes no further reference to the blood content of the tumour. In his later work on the growth curves of ascites tumours (Klein and Révész, 1953) he states that there was " often a slight admixture of erythrocytes " in the Ehrlich ascites carcinoma, but this was not studied further and in some experiments blood stained fluids were not investigated. Kun, Talalay and Williams-Ashman (1951) say, with regard to this tumour, " Characteristically, the fluid is milky white, has a tendency to clot, and occasionally is grossly hemorrhagic. Fluids containing more than about 15 per cent erythrocytes of the total cells were discarded ".

The present experiment shows that the amount of blood is related to the survival time, a highly significant negative correlation being present between the blood volume/ml. ascites and the survival time. It may be argued that the blood volume might remain constant while the tumour volume increased, giving in itself a negative correlation. But if the percentage blood/ml. is multiplied by the volume of tumour ascites present, and this figure (the total blood present) is correlated to the survival time, the negative correlation is still highly significant. Referring this finding back to the two groups discussed previously we find that the blood content of the tumours in groups a and b differs to a very highly significant extent, the tumours of the mice with a short survival time containing more blood than those of mice surviving longer.

Apitz (1934) tested the idea that haemorrhage into the solid Ehrlich carcinoma might be due to anaphylaxis but obtained negative results on a small series of stock mice. From the results of the present experiment it is possible to say that the blood content of the Ehrlich ascites carcinoma is related to the survival time

and is an expression of the finding that the mice react to the tumour in two ways but it is not possible to say why they do so. Further experiments on this question are in progress.

SUMMARY

Twenty-five male and 25 female mice were injected intraperitoneally with the same cell dose of Ehrlich's ascites carcinoma. This was taken from one mouse. It was found that the mice fell into two distinct groups as regards survival time, although the survival time was not influenced by the sex or weight of the mouse. The amount of tumour was less in the smaller mice. A positive correlation was found between the survival time and the final tumour volume, and a negative correlation between the survival time and the volume of blood in the tumour.

REFERENCES

AHLSTRÖM, C. G. AND ISING, U.—(1955) *Acta path. microbiol. scand.*, **36**, 415.
APITZ, K.—(1934) *Z. Krebsforsch.*, **40**, 50.
KARNOFSKY, D. A.—(1953) 'Experimental Cancer Chemotherapy', 635. In: 'The Physiopathology of Cancer', ed. Homburger and Fishman. New York (Paul B. Hoeber Inc.).
KLEIN, G.—(1950) *Cancer*, **3**, 1052.
Idem. and RÉVÉSZ, L.—(1953) *J. nat. Cancer Inst.*, **14**, 229.
KUN, E., TALALAY, P. AND WILLIAMS-ASHMAN, H. G.—(1951) *Cancer Res.*, **11**, 855.
LETTRÉ, H.—(1941) *Z. physiol. Chem.*, **271**, 192.
LOEWENTHAL, H. AND JAHN, G.—(1932) *Z. Krebsforsch.*, **37**, 437.

DEVELOPMENT OF MOUSE ASCITES TUMOR SUBLINES RESISTANT TO TRIETHYLENE MELAMINE

ANNA MARIA WILLIAMS AND ELAINE JORANGER

From the Department of Medicine, University of Wisconsin Medical School, Madison, Wisconsin, U.S.A.

Received for publication February 28, 1961

REPORTS of experimental tumors resistant to various nucleic acid, amino acid, and folic acid antagonists used in cancer chemotherapy are now fairly numerous (Welch, 1959), and several mechanisms of resistance have been demonstrated by comparing resistant tumors with their corresponding sensitive lines. Much less work has been done with tumors resistant to alkylating agents. Jackson (1954) has reported a subline of the Walker rat carcinoma resistant to triethylene melamine (TEM), and Yoshida (1959), a nitromin-resistant subline of the Yoshida rat ascites sarcoma. Several workers have studied rat tumors (Gorozhansky, 1959; Kurita *et al.*, 1959; Oboshi, 1959) and Ehrlich mouse ascites cells (Inoue *et al.*, 1958) resistant to nitrogen mustard derivatives.

Mouse ascites tumors are convenient tools for *in vivo* and *in vitro* studies on the mechanism of action of chemotherapeutic agents and on possible differences between sensitive and resistant cells. It seemed worthwhile to attempt to develop sublines of mouse ascites tumors resistant to alkylating agents commonly used in treatment of human leukemias. We were particularly interested in testing possible differences in nucleic acid metabolism, and used the Ehrlich carcinoma and Sarcoma 180 (S180) ascites tumors, since considerable information on their nucleic acid metabolism was already available (Hartman and Buchanan, 1959). The effects of two alkylating agents, TEM and busulfan, on these ascites tumors were investigated, and sublines resistant to TEM were developed by consecutive passages of tumor cells in animals given intraperitoneal injections of this drug.

MATERIALS AND METHODS

Mice.—Female white Swiss mice, 20–25 g., were obtained from A. R. Schmidt & Co., Madison, Wisconsin, and were watered and fed Rockland rat diet *ad libitum*. Unless otherwise noted, there were 10 mice of comparable weight in each experimental group. Survival times were recorded to the nearest half day.

Transplanatation of tumors.—Ascites tumor cells were transplanted by withdrawing the ascites fluid from a donor mouse bearing a 7-day tumor growth. The fluid was centrifuged for 5 minutes in a clinical centrifuge at 1470 × g, the supernate was decanted, and the cells were diluted 1:20 with isotonic saline. Each animal received 0·2 ml. of the cell suspension (10^7 cells).

Injection of drugs.—Drugs were injected intraperitoneally beginning 24 hours after tumor implantation, and continued at 24-hour intervals when 3 or 4 injections were given or at a 48-hour interval when 2 injections were given. Drug dosages of TEM designated A–F in the Tables are as follows: A, 4 mg./kg., 1×

B, 2 mg./kg., 1×; C, 2 mg./kg., 2×; D, 1·3 mg./kg., 3×; E, 1 mg./kg., 4×; F, 0·8 mg./kg., 3×. Control groups received injections of saline. Drugs were dissolved in saline and injected in volumes of 0·2 ml. TEM or 0·5 ml. busulfan. Solutions of TEM were prepared from the powder for each series of injections and thus held in the refrigerator not longer than 3 days. TEM was obtained from Lederle Laboratories and the "Myleran" brand of busulfan from Burroughs Wellcome & Co.

The following dosages of drugs were administered in the experiment described in Table V. 6-Mercaptopurine: 30 mg./kg., 1×/day for 6 days; thioguanine: 0·5 mg./kg., 2×/day for 6 days; azaserine: 0·2 mg./kg., 2×/day for 6 days; 5-fluorouracil: 20 mg./kg., 1×/day for 7 days; azauridine: 1·5 mg./kg. 1×/day for 6 days; amethopterin: 0·25 mg./kg., 1×/day for 6 days.

Measurement of packed cell volume.—Packed cell volume was measured by collecting the ascites fluid (containing ascites cells and any infiltrated blood cells) in a graduated conical tube and centrifuging for 5 minutes in a clinical centrifuge at 1470 × **g**.

RESULTS

Effect of busulfan

Treatment with 20 mg./kg. busulfan at 12-hour intervals for 3–5 days resulted in survival times equal to those of untreated control groups, and reduced packed cell volumes of the Ehrlich tumor by about 70 per cent and of the S180 tumor by about 50 per cent. Lower dose regimens did not significantly prolong survival times, and thus no further studies were done with this drug.

Effect of triethylene melamine

Table I shows the effect of various dose regimens of TEM on Ehrlich and S180 ascites cells. It was found that 4 mg./kg. body weight was the maximum dose that could be given in one injection if the animals were to survive as long as

TABLE I.—*Effect of TEM on Ehrlich and Sarcoma* 180 *Ascites Cells*

Treatment*	Days survival		ml. Packed cell volume	
Ehrlich				
Control	11·9 ± 1·0†	(7·5–15·5)‡	2·7 ± 0·23†	(1·3–4·0)‡
A	14·6 ± 1·0	(10–20·5)	0·2 ± 0·09	(0–0·9)
B	16·8 ± 1·4	(10·5–25)	2·9 ± 0·36	(0·9–4·0)
C§	12·8 ± 0·7	(8·5–16)	0·1 ± 0·01	(0–0·3)
D§	16·4 ± 0·9	(10–21·5)	0·2 ± 0·08	(0–0·7)
E	15·5 ± 0·5	(13·5–18·5)	1·8 ± 0·23	(0·6–2·5)
F	22·7 ± 2·4	(13·5–33·5)	2·2 ± 0·49	(0·4–4·0)
Sarcoma 180				
Control	12·1 ± 0·6	(9–14)	2·3 ± 0·10	(2·0–2·8)
A	13·2 ± 1·6	(8–22)	0·3 ± 0·08	(0–0·9)
B	18·0 ± 1·1	(9–21)	1·8 ± 0·23	(1·0–3·2)
C§	18·8 ± 0·7	(8–21)	0·4 ± 0·06	(0·1–0·6)
D	17·7 ± 1·0	(11–20·5)	0·3 ± 0·06	(0·1–0·6)
E	19·3 ± 1·2	(16–21·5)	0·6 ± 0·15	(0·2–1·7)

* Drug dosages are given in Methods.
† Standard deviation of the mean.
‡ Range of values.
§ Group contained 9 mice.

7 days. Ruvidic, Mathe and Bernard (1958) have reported that a single injection of 5 mg./kg. body weight TEM induced aplasia of the bone marrow in mice with death of all animals within 6 days. Survival times of mice bearing the S180 tumor were prolonged under all dose regimens except A, and those of mice with the Ehrlich tumor, by all but C. The relatively non-toxic treatment F almost doubled the survival time of mice with the latter tumor.

Eight of the mice receiving a total of 4 mg./kg. in 1, 2, and 3 doses (treatments A, C, D) showed no tumor cells at death (10–14·5 days), but this drug concentration was too toxic for the mice to live indefinitely and be labeled as " cures ". Since drug toxicity was a factor in the survival times of animals receiving the higher doses of TEM, packed cell volumes were also used to compare the effectiveness of this drug against sensitive lines of ascites cells *versus* resistant sublines. This measurement, of course, does not distinguish differences in cell size. When smears were made at the time of death of the tumor-bearing animals, there were no noticeable size differences between sensitive line and its resistant sublines. Ascites cells of treated animals did not differ noticeably from those of untreated animals when death occurred after about 12 days. When animals died before 10–12 days with small volumes of tumor cells (*i.e.*, from drug toxicity), the cell population was not so homogenous in size, but the variation included both larger and smaller cells.

Development of sublines resistant to triethylene melamine

Both Ehrlich and S180 tumors were treated with 4 mg./kg. one time (Treatment A), and pooled cells from several mice were transplanted at intervals of 7 to 21 (usually 14) days. The 14th Ehrlich and 28th S180 transplants were used for the experiments of Table II. After the 22nd generation, another series was begun

TABLE II.—*Effect of TEM on Sublines of Ascites Cells Previously Treated with TEM*

Treatment*	Days survival	ml. Packed cell volume
Ehrlich Subline Treatment A		
Control†	16·6 ± 1·5‡ (9–25)§	2·6 ± 0·18‡ (1·5–3·2)§
A	15·5 ± 0·5 (12·5–19)	1·1 ± 0·36 (0·1–2·7)
B	12·7 ± 1·7 (8–22·5)	1·0 ± 0·09 (0·8–1·5)
C	18·3 ± 1·6 (10–24)	2·2 ± 0·33 (0·8–4·0)
Sarcoma 180 *Subline Treatment A*		
Control	15·1 ± 1·3 (9·5–21)	2·9 ± 0·32 (1·5–4·5)
A	16·3 ± 1·2 (10–21)	1·1 ± 0·19 (0·2–1·9)
B	15·2 ± 0·7 (14–19)	2·4 ± 0·32 (1·1–4·0)
C	18·5 ± 1·1 (15–26·5)	2·5 ± 0·34 (0·9–4·4)
D	15·7 ± 0·5 (14–19)	2·4 ± 0·41 (0·4–4·1)
E	17·4 ± 0·7 (15–22)	2·5 ± 0·41 (0·7–4·6)
Sarcoma 180 *Subline Treatment D*		
Control	17·3 ± 0·9 (11·5–20)	2·2 ± 0·18 (1·5–3·0)
A	16·1 ± 1·5 (9·5–22)	1·3 ± 0·36 (0·2–4·5)
B	16·9 ± 0·5 (13–18)	3·3 ± 0·24 (2·2–4·5)
C	21·3 ± 1·2 (19–29)	2·6 ± 0·34 (1·2–4·0)
D	18·6 ± 0·6 (16–22)	2·7 ± 0·24 (1·3–3·5)
E	18·9 ± 1·0 (15–23)	2·6 ± 0·28 (1·8–4·1)

* Drug dosages are given in Methods.
† Group contained 9 mice.
‡ Standard deviation of the mean.
§ Range of values.

from these resistant transplants to be treated with 1·3 mg./kg. 3 times (Treatment D), a more convenient dose regimen from the standpoint of toxicity and numbers of mice to be routinely transplanted. The 4th transplant generation of the S180 Treatment D subline was used in the experiment of Table II.

A comparison of control groups in Tables I and II shows that mice bearing the resistant sublines had longer survival times. The experiment with the Ehrlich resistant subline was run at the same time as that with the Ehrlich sensitive line in Table I, and average survival times were 16·6 (resistant) and 11·9 (sensitive) days. Control groups of the S180 sensitive line and Treatment D subline were run at the same time as the experiment with the S180 Treatment A subline shown in Table II, with the following survival times and packed cell volumes: sensitive, 12·1 $\pm$ 1·2 days and 2·4 $\pm$ 0·29 ml.; Treatment D subline, 14·8 $\pm$ 0·9 days and 2·2 $\pm$ 0·25 ml. Since packed cell volumes at death did not differ greatly, the resistant tumors evidently grow more slowly than the sensitive lines.

Treatment B prolonged the average survival times of mice bearing the sensitive tumors (Table I), but not of those bearing the resistant sublines (Table II). Treatment C prolonged the average survival times of mice bearing the resistant S180 sublines, but the average packed cell volumes measured at death were much greater than that of the sensitive line given Treatment C. The data in Table III

TABLE III.—*Relative Packed Cells Volumes of Sensitive and Resistant Cells when Survival Times were Equal*

Line of cells	Treatment*	Days survival	ml. Packed cell volume
Ehrlich Sensitive			
	None	13·5, 14	3·3, 3·0
	C	13·5, 14	0, <0·1, 0·1
Ehrlich Subline Treatment A			
	None	15·5, 16·5	1·5, 2·8
	C	14	3·0, 3·4
S180 Sensitive			
	None	14	2·0, 2·0, 2·2
	C	19, 20	0·2, 0·3, 0·5
	D	14, 19, 20	0·1, 0·3, 0·2, 0·3
S180 Subline Treatment A			
	None	15	2·5, 3·9
	C	15	3·4
	D	15	2·1, 2·4, 3·8
S180 Subline Treatment D			
	None	19, 20	1·5, 2·2, 2·8, 2·0
	C	19	2·4, 2·8, 3·2, 4·0
	D	20	2·0, 3·3, 3·4, 3·5

* Drug dosages are given in Methods.

have been arranged to show this difference in packed cell volumes. Some values from mice of each subline are given to compare control group *versus* treated groups with similar survival times, and sensitive *versus* resistant sublines with similar survival times. In the latter comparison, it should be remembered that the resistant tumors are slower growing. Treated mice of resistant sublines had cell volumes equal to or greater than those of their controls, in contrast to results with sensitive lines.

To determine if the resistance was stable in the absence of TEM, a series was started from the 27th transplant generation of the S180 Treatment A subline and carried without the drug. Tests of the 8th transplant generation (Table IV) showed

TABLE IV.—*Effect of TEM on Resistant Subline of Sarcoma* 180 *Carried Without TEM for Eight Transplant Generations*

Treatment*	Days survival		ml. Packed cell volume	
Control	16·7 ± 0·9†	(10–20)‡	3·1 ± 0·14†	(2·4–3·7)‡
B	15·9 ± 0·6	(13·5–19)	3·3 ± 0·30	(1·5–5·1)
C	18·9 ± 0·9	(16·5–22)	1·8 ± 0·34	(0·8–3·4)
D	19·1 ± 1·1	(17–27)	3·1 ± 0·31	(2·0–4·5)

* Drug dosages are given in Methods.
† Standard deviation of the mean.
‡ Range of values.

that the tumor was still resistant, with both the slower growth characteristic and the proliferation in the presence of TEM shown by the treated resistant subline.

*Effect of antimetabolites on S*180 *cell populations sensitive and resistant to TEM*

Table V compares the action of various antimetabolites known to affect nucleic acid metabolism on the S180 tumors sensitive and resistant to TEM. All

TABLE V.—*Effect of Various Antimetabolites on Sarcoma* 180 *Ascites-cell Populations Sensitive and Resistant to TEM*

	S180 sensitive to TEM		S180 resistant to TEM	
Drug*	Days survival	ml. Cells	Days survival	ml. Cells
Control	11·7 ± 0·6† (9–15)‡	2·3 ± 0·25† (1·2–3·5)‡	18·3 ± 0·9 (13–21)	1·4 ± 0·15 (0·5–2·2)	
6-Mercaptopurine	13·0 ± 0·9 (8–15)	1·2 ± 0·15 (0·3–1·8)	51·6 ± 3·5 (25–56)§	0·13 ± 0·09 (0–0·8)	[6, 0]‖
Thioguanine	15·7 ± 1·0 (10–21·5)	1·7 ± 0·37 (0·3–4·1)	56 ± 0	0	[8, 2]
Azaserine	18·6 ± 0·6 (13·5–20)	3·9 ± 0·36 (1·6–6·0)	51·6 ± 2·7 (30–56)	0·17 ± 0·09 (0–0·7)	[6, 1]
5-Fluorouracil	18·8 ± 0·8 (13·5–22)	1·8 ± 0·28 (0·5–3·2)	55·2 ± 0·8 (48–56)	0·48 ± 0·30 (0–2·8)	[6, 1]
Azauridine	21·4 ± 2·0 (13–34)	1·0 ± 0·24 (0·2–2·4)	39·4 ± 4·8 (19–56)	1·5 ± 0·30 (0·3–5)	[2, 0]
Amethopterin	23·3 ± 0·9 (18–27)	1·6 ± 0·39 (0·1–3·5)	41·4 ± 2·7 (30–56)	0·73 ± 0·33 (0–1·8)	[2, 0]

* Drug dosages are given in Methods.
† Standard deviation of the mean.
‡ Range of values.
§ Group contained 9 mice.
‖ The first figure in brackets is the number of mice without ascites cells or solid tumors; the second figure is the number with no ascites cells or fluid but with one solid tumor in the peritoneal cavity.

6 drugs had a greater chemotherapeutic effect on the resistant Treatment A subline, and a number of animals bearing this subline were free of tumor cells when killed on the 56th day. These have been considered as 56-day survivors in the calculation of average survival time. A few animals had no ascites cells or fluid but exhibited a single solid tumor within the peritoneal cavity.

The slower growth of the resistant subline must be considered in evaluating these results, and since both tumor lines were treated with drug 24 hours after transplantation, it might be argued that the resistant tumor was in an earlier stage of growth. However, the relative increase in sensitivity to these antimetabolites displayed by the resistant tumor was not the same for all the drugs. Thioguanine and 6-mercaptopurine were the least effective drugs, and amethopterin and azauridine the most effective, in prolonging survival times of mice bearing the tumor sensitive to TEM. With the tumor resistant to TEM, thioguanine was the most effective drug and amethopterin and azauridine were the least effective; 6-mercaptopurine was more effective than the latter two drugs.

DISCUSSION

The only other report of a tumor resistant to TEM of which we are aware is that of Jackson (1954), who described the development of Walker carcinosarcoma solid tumors. These grew after a latent period of about 2 weeks following treatment with 0·2 mg./kg. TEM daily for 8 days. A resistant tumor was maintained by transplantation with and without drug, and the line carried without drug was still resistant after about 50 transplant generations. Interestingly, in untreated animals the resistant rat tumor grew more rapidly than the original tumor, in contrast to our resistant tumor which grew more slowly. From various considerations, Jackson concluded that the resistant tumor had arisen from the survival of a small number of cells which were naturally resistant to TEM, but that the contribution of the mutagenic effects of the drug remained to be investigated.

The present work has demonstrated the feasibility of developing mouse ascites tumors resistant to TEM. We had been primarily interested in obtaining a stable tumor resistant to this alkylating agent for biochemical studies, and our work does not bear upon the question of selection or mutation as the mechanism by which resistance was developed. However, our resistant subline provides an example of a tumor which after treatment with one drug was markedly more susceptible to certain other drugs. The increased sensitivity of the resistant S180 subline to a number of antimetabolites known to affect nucleic acid metabolism is best explained by the selection of a population with altered pathways for nucleic acid biosynthesis, and this supposition is under investigation.

SUMMARY

Sublines of two mouse ascites tumors, the Ehrlich carcinoma and Sarcoma 180, exhibiting resistance to triethylene melamine were developed by consecutive passage of cells in mice treated with triethylene melamine. The change was found to be heritable, and stable for at least 8 transplant generations in the absence of the drug. The S180 resistant subline showed increased sensitivity to azaserine, amethopterin, 5-fluorouracil, azauridine, thioguanine, and 6-mercaptopurine. Doses of the latter two drugs which prolonged the survival times of the sensitive subline by less than 35 per cent, with all animals dying, prolonged that of the resistant subline by at least 180 per cent, with more than half of the animals free from tumor cells when killed at 56 days.

This research was supported by a grant from the National Institutes of Health, U.S.P.H.S. (CY–5936), and the E. K. Holz fund.

REFERENCES

Gorozhanskaya, E. G.—(1959) *C.R. Acad. Sci., U.R.S.S.*, **127**, 911.
Hartman, S. C. and Buchanan, J. M—(1959) *Annu. Rev. Biochem.*, **28**, 365.
Inoue, A., Koseki, Y., Onoe, P. and Tsukada, H.—(1958) *Gann* **49** suppl., 63.
Jackson, H.—(1954) *Brit. J. Cancer*, **8**, 336.
Kurita, S., Takemura, C., Hoshino, A. and Kimura, K.—(1959) *Gann* **49**, suppl., 64.
Oboshi, S.—(1959) *Gann*, **50**, 147.
Ruvidic, R., Mathe, G. and Bernard, J.—(1958) *Rev. franç. Etud. clin. biol.*, **3**, 159.
Welch, A. D.—(1959) *Cancer Res.*, **19**, 359.
Yoshida, T.—(1959) *Gann*, **49**, suppl., 62.

THE INACTIVATION BY ORGANIC SOLVENTS AND DETERGENTS OF PARTIALLY PURIFIED ROUS I VIRUS PREPARATIONS

H. A. DRAYTON

From the British Empire Cancer Campaign Unit, A.R.C. Poultry Research Centre, West Mains Road, Edinburgh, 9

Received for publication March 23, 1961

In experiments on the action of bile salts on viruses, Smith (1939) found that crude filtrates of Rous I sarcoma were inactivated by a 5 per cent solution of sodium desoxycholate. Burnet and Lush (1940) were able on the basis of their investigations on the action of sodium desoxycholate and sodium laurylsulphate on a number of viruses, to arrange these in a series of graded susceptibility, which was the same for the two types of chemical. Andrewes and Horstmann (1949) in their study of the sensitivity of 25 viruses to diethyl ether, found that crude filtrates of Rous I sarcoma, active initially in a dilution of 1 : 1,000, were inactivated to an extent such that only the undiluted filtrates were infective. They drew attention not only to the close correlation between the sensitivity of viruses to ether and to bile salts and synthetic detergents, but also to the fact that a virus like Rous I sarcoma wihch is sensitive to these reagents is also extremely labile at ordinary temperatures. Recently, Guerritore (1957, 1958) has claimed that although Rous I sarcoma virus preparations are inactivated by both anionic and cationic detergents, that the inactivation produced by the latter is reversible by the addition of either heparin or ribonucleic acid (yeast RNA).

The experiments described in this paper are concerned with the treatment of partially purified Rous I virus preparations with organic solvents, bile salts and synthetic detergents in an attempt to correlate decrease in infectivity with virus material liberated and detected by u.v. spectrophotometric analysis. This technique had previously been found useful in a study of the effect of incubation on fowl tumour viruses (Drayton, 1960).

MATERIALS AND METHODS

The method of preparing partially purified Rous I virus and GRCH 16 (Peacock and Peacock, 1953) particulates using fractional centrifugation and enzyme treatment, and the day-old chick titration method for titrating activity have been previously described (Carr and Harris, 1951 ; Bather, 1953). The chicks used for bioassay were all from the highly susceptible inbred line of Brown Leghorns at the Poultry Research Centre.

Organic solvents.—These included ethyl alcohol, diethyl ether (pure anaesthetic), chloroform and ethylene glycol.

Bile salts.—0·25 per cent solutions of sodium desoxycholate, sodium glycocholate and sodium taurocholate were employed.

Synthetic detergents.—In the case of Cetavlon (cationic detergent) 2 mg. ml. of purified virus preparation was used ; with sodium lauryl sulphate (anionic detergent), 0·4 mg. ml.

EXPERIMENTAL

In each experiment the weights of GRCH tumour from which the cell particulate suspensions were derived, were equal to the weights of Rous I tumour (about 50 g.) used in the preparation of partially purified virus. The total nitrogen content of GRCH 16 particulates has been found to be 0·34 mg. per g. of wet tumour compared with 0·037 mg. per g. in the case of Rous I (Bather, 1953).

The experimental technique was essentially the same for each of the three types of reagent. Equal volumes of partially purified Rous I virus preparation, the infectivity of which had previously been determined by titration in young chicks, were diluted 1 : 10 with each of the reagents and carefully mixed. After centrifugation at an angle at 15,000 *g* for 55 minutes at 0° C., the supernatant in each case was diluted appropriately and examined in the u.v. spectrophotometer (Unicam SP 500) at wavelengths between 2400 Å and 3000 Å. The blank solution was the reagent used for treatment of the virus. Optical density was plotted against wavelength. The precipitate in each case was resuspended in McIlvaine's phosphate-citric buffer, pH 7·2 and its infectivity determined by the bioassay procedure mentioned above.

GRCH particulate suspensions were treated with diethyl ether, sodium desoxycholate and cetavlon respectively.

Aseptic technique was observed throughout ; tests for the presence of contaminating bacteria, using nutrient agar slopes incubated at 37° C., were negative.

The results of many experiments designed in the manner described above were consistent.

RESULTS

The preparations of Rous virus were inactivated by all the reagents tested—organic solvents, bile salts and detergents. Only with sodium lauryl sulphate was a small residual infectivity retained ; in a typical experiment, for example, a virus preparation with an initial infectivity (m.i.d. /g. tumour) of $10^{5.3}$ (*i.e.* 3 out of 4 positive inoculations at 10^{-5} dilution) still had a titre of 10^{1} (*i.e.* 2 out of 4 positive inoculations at 10^{-1} dilution) after treatment with this detergent. Complete loss of infectivity resulted from the other treatments.

In every case the loss of infectivity was accompanied by shedding of material which absorbs in the ultra-violet between 2700 Å and 2800 Å and is very likely protein (Fig. 1, 2 and 3). In no case was any intense absorption peak observed at 2600 Å. Part of the u.v. absorption curve of the water soluble Rous virus fraction (?RNA+) extracted with phenol is included in Fig. 2 for comparison. It is inferred therefore that the presence of significant amounts of nucleic acid in the experimental supernatants is unlikely.

GRCH 16 particulate suspensions treated in precisely the same manner with a representative of each of the three types of reagent, did not yield material in the supernatants with the same absorption character in the range examined (Fig. 1, 2 and 3).

DISCUSSION

Rous I virus is known to consist of protein, ribonucleic acid (RNA) and lipid, a large proportion of which is in the form of phospholipid (Claude, 1935 ; Bather, 1957). Some of the protein is bound to RNA in an electron dense " nucleoid " at

the centre of the virus particles associated with Rous I sarcoma ; two membranes can also be clearly distinguished around the nucleoid (Oberling, Bernhard and Vigier, 1957 ; Epstein, 1958).

Bather (1957) has shown that the RNA associated with partially purified preprations of Rous I sarcoma virus can be quantitatively estimated using 10 per cent NaCl solution and ultra-violet spectrophotometry. From a series of 38 Rous I sarcoma virus preparations with a range of infectivity (m.i.d. g./tumour) of $10^{1\cdot2}$–$10^{6\cdot5}$ he extracted an average of 1·42 per cent $\pm$ 0·37 RNA (values ranging from 0·64–2·12 per cent). Further work (Bather, 1958) provided evidence of a

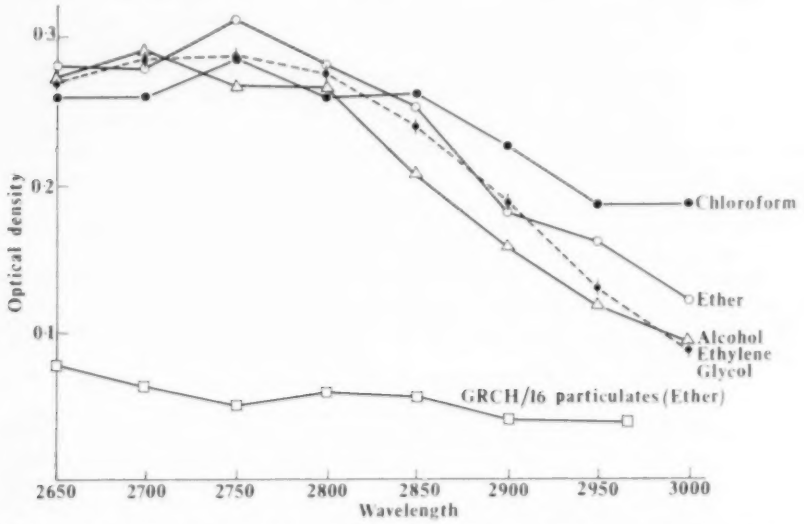


FIG. 1.—Ultraviolet absorption spectra between 2650 Å and 3000 Å, of supernatant after treatment of preparations with organic solvent indicated.

positive correlation between the infectivity of a partially purified tumour virus preparation and its RNA content. If the graph published, which shows this relationship, is transposed to a log-log scale than a visually better regression line can be fitted. From such a log plot, it can be deduced that the percentage of RNA which would correspond, on the average, to the infectivity of $10^{5\cdot3}$ of our virus preparation is between 1 and 1·4 per cent. The detection of this amount of RNA is just within the limits of sensitivity of the u.v. spectrophotometry technique.

The fact that treatment of Rous I virus preparations with a number of lipid solvents yields detectable amounts of "protein-like" material indicates that some portion of the protein moiety of Rous I virus particles is bound to lipid in lipid-protein complexes. The absence of detectable nucleic acid in the supernatants after any of the experimental treatments suggests that the lipid-protein units are external to the nucleoid and are probably integrated into the two outer membranes observed in electron micrographs of Rous I virus particles.

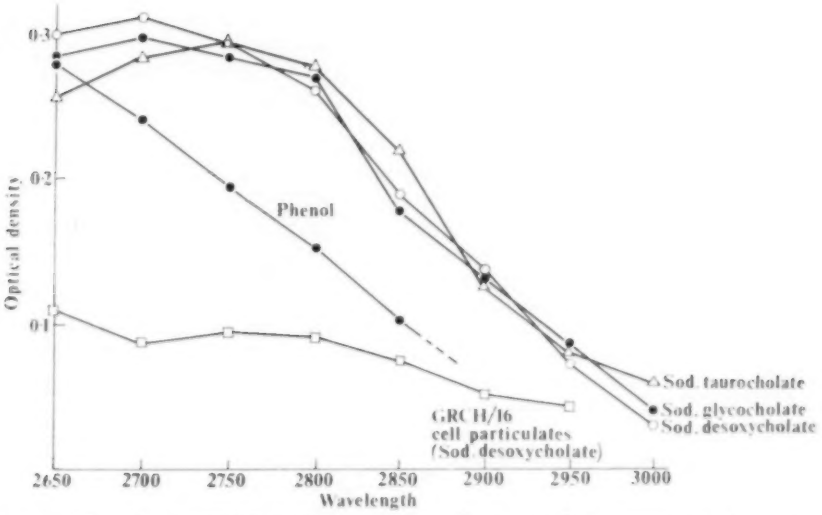


FIG. 2.—Ultraviolet absorption spectra between 2650 Å and 3000 Å of supernatant after treatment of preparations with bile salt indicated. (Portion of absorption curve of water soluble fraction of Rous virus (?RNA +) extracted with phenol inserted for comparison.)

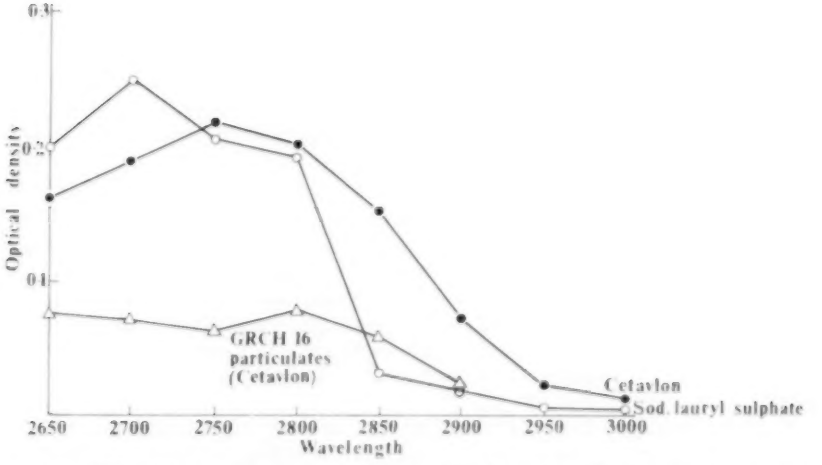


FIG. 3.—Ultraviolet absorption spectra between 2650 Å and 3000 Å, of supernatant after treatment of preparations with synthetic detergent indicated.

Detergents, both synthetic ones like Cetavlon and sodium lauryl sulphate and biological ones like the bile salts, are capable of denaturing proteins by disrupting electrovalent linkages. Results reported here show that Rous I virus is extremely sensitive to these reagents and it can be concluded therefore that electrovalent linkages must play an important part in the structure of the extra-nucleoid virus protein.

Very little is known with any certainty about the types of linkage involved between the lipid and protein in lipoproteins. But Dervichian (1949) has presented evidence and arguments that the basic unit of all lipoproteins is an ionic compound of lipid and protein, and it is the phospholipids by virtue of their residual acidic and basic groupings which can combine with proteins by primary valency forces. As mentioned above a large proportion of the lipid in Rous I virus is known to be phospholipid.

The supernatants from GRCH 16 particulate suspensions (derived in each experiment from tumour equal in weight to the Rous I sarcoma, and treated with a representative of each of the three types of reagent) did not contain material with the same sort of u.v. absorption character. From the comparative figures for nitrogen content of Rous I and GRCH 16 (Bather, 1953) quoted above there would be about ten times the number of GRCH particles as for Rous. It can be concluded therefore that the protein-like material detected in these experiments derived from constituent virus protein rather than from associated host material.

A similar " leakage " of protein-like material from the virus particles of Rous I, MH_2 (Begg, 1927) and PRC_4 (Carr and Campbell, 1958) partially purified virus preparations, after incubation at 37° C. for periods up to 24 hours has been previously reported (Drayton, 1960). It must be contrasted with the effect of phenol on partially purified avian tumour virus preparations—a water soluble fraction is obtained which gives a single smooth absorption curve in the ultra-violet with an intense peak at 2600 Å and is probably RNA. The degraded virus which sediments on high speed centrifugation is completely non-infective, as are the majority of the RNA fractions so far tested (unpublished data).

It would appear therefore that the infectivity of the tumour viruses requires not only an intact RNA-protein component but also the integrity of at least some of the protein bound to lipid in the extra-nucleoid virus material.

SUMMARY

1. Treatment of partially purified Rous I virus preparations with a number of organic solvents, synthetic detergents and bile salts resulted in a loss of infectivity, which was complete in every case, except sodium lauryl sulphate.

2. The loss of infectivity after each treatment was accompanied by a liberation into the supernatant after sedimentation, of material which absorbed strongly in the region 2700 Å–2800 Å and is very likely protein. Similar material was not detected in GRCH 16 particulate suspensions after the same experimental treatments.

3. It is suggested that the infectivity of Rous I virus requires not only an intact RNA protein component but also the integrity of at least some of the protein bound to lipid in the extra-nucleoid virus material.

All expenses in connexion with this work were borne by the British Empire Cancer Campaign.

REFERENCES

ANDREWES, C. H. and HORSTMANN, D. M.—(1949) *J. gen. Microbiol.*, **3**, 290.
BATHER, R.—(1953) *Brit. J. Cancer*, **7**, 492.—(1957) *Ibid.*, **11**, 611.—(1958) *Ibid.*, **12**, 256.
BEGG, A. M.—(1927) *Lancet*, i, 912.
BURNET, F. M. AND LUSH, D.—(1940) *Aust. J. exp. Biol. med. Sci.*, **18**, 141.
CARR, J. G. AND CAMPBELL, J. G.—(1958) *Brit. J. Cancer*, **12**, 631.
Idem AND HARRIS, R. J. C.—(1951) *Ibid.*, **5**, 83.
CLAUDE, A.—(1935) *J. exp. Med.*, **61**, 41.
DERVICHIAN, D. G.—(1949) *Disc. Faraday Soc.*, No. 6, 7.
DRAYTON, H. A.—(1960) *Brit. J. Cancer*, **14**, 306.
EPSTEIN, M. A.—(1958) *Nature, Lond.*, **181**, 1808.
GUERRITORE, D.—(1957) *Z. Krebsforsch.*, **61**, 649.—(1958) *Nature, Lond.*, **181**, 419.
OBERLING, C., BERNHARD, W. AND VIGIER, P.—(1957) *Ibid.*, **180**, 386.
PEACOCK, P. R. AND PEACOCK, A.—(1953) *Brit. J. Cancer*, **7**, 120.
SMITH, WILSON.—(1939) *J. Path. Bact.*, **48**, 557.

THE EFFECTS OF DEXTRAN-SALINE (" DEXTRAVEN ") UPON CELLS CULTIVATED *IN VITRO*. THE RESPONSE OF ACTIVELY GROWING HeLa CARCINOMA CELLS

A. K. POWELL

From the Department of Experimental Pathology, Mount Vernon Hospital, Northwood, Middlesex

Received for publication April 13, 1961

TRYPSIN dissolved in saline solution is commonly used to prepare suspensions of viable cells from small pieces of tissue or monolayer cultures. In practice not all the discrete cells are viable. In an attempt to decrease cell mortality it was proposed to use trypsin dissolved in a non-protein blood plasma substitute. Preliminary trials of the dextran-saline plasma substitute " Dextraven " (Benger Laboratories) were made on HeLa carcinoma cells, at the incomplete monolayer stage of growth, cultured in 8-oz. flat bottles. The cultures were treated with stock " Dextraven " for 6 hours, the bottles repeatedly rinsed with buffered saline solutions, and normal culture medium then supplied. The cultures were 7 days old at the time of treatment and last fed 4 days prior to treatment so that the medium was almost exhausted.

After treatment the cultures were fed at intervals of 3 or 4 days and the cells observed *in situ* for cytotoxic effects. Little definite evidence of damage to the attached cells was observed. The gross morphology of the treated cells remained normal, although there were subtle alterations in the appearance of the protoplasm and the pH of the medium did not become acid. Observed at a magnification of $\times 150$ the cells *in situ* seemed to be intact; the nuclei, nuclear membranes and nucleoli were well defined and the cells retained the usual extended cytoplasmic processes. The treated cells did not proliferate or acidify the culture medium. This inert state of the cells persisted for several months.

Concurrently with these observations the effects of an iron-dextran complex (" Imferon ", Benger Laboratories) on chick embryo fibrocytes cultivated *in vitro* were being studied (Powell and Turner, unpublished work). Richmond (1957, 1959) and Haddow and Horning (1960) found that " Imferon " was carcinogenic to laboratory animals under certain conditions. In view of the possible involvement of dextran in the carcinogenicity of " Imferon " its unusual effects on HeLa cells were further investigated.

MATERIALS AND METHODS

Dextraven.—The undiluted standard proprietary preparation—6 per cent dextran in 0·9 per cent w/v sodium chloride solution—was used in the experiments. 0·9 per cent sodium chloride solution was used as a control medium.

HeLa carcinoma cultures.—Stock cultures of HeLa cells were serially maintained in 8-oz. soft glass flat bottles. They were fed on a growth medium essentially that described by Pereira and Kelly (1957). The Gey's balanced saline contained 0·125 g. of $NaHCO_3$ per litre. Yeast extract was prepared by heating

a solution of dried yeast at 99° C. for 20 minutes, allowing the undissolved residue to sediment during rapid cooling and Seitz filtration of the supernatant solution The lactalbumin hydrolysate solution was also Seitz filtered.

Stock cultures were divided weekly into 2 or 3 sub-cultures as required. Measured volumes of fresh medium were added to the parent cultures, the cells detached from the glass at the monolayer stage of growth by gentle pipetting and the cell suspension distributed into fresh culture bottles.

Experimental cultures.—These were prepared from cell suspensions in hexagonal roller-tubes, each holding 6 rectangular No. 2 coverslips, about a week before treatment. At this latter time the cells occupied about a quarter of the total area of a coverslip, in both sheet and open growth habits. By this time most of the cells damaged during subculturing had been removed during routine changes of medium. The cultures were fed 24 hours before treatment. Before the addition of " Dextraven " the roller-tubes were rinsed twice with Gey's balanced saline solution. During the rinses the coverslips were individually loosened from the walls of the roller-tubes. The tubes were then given a preliminary rinse with " Dextraven " and the coverslips again loosened. Finally, about 3·5 ml. of " Dextraven " was placed in each tube. Both the Gey's solution and the " Dextraven " were pre-warmed to 37° C. and the operations done as quickly as possible. Untreated control cultures given only growth medium and cultures given physiological saline solution as a control to the " Dextraven " solution were comparably treated. At the end of the period of treatment the procedure of changing the solutions was reversed and the cultures fed on normal medium. All cultures were incubated at 37° C.

Coverslips bearing the carcinoma cells were fixed in Heidenhain's " Susa " mixture immediately after treatment and at intervals thereafter, stained with Ehrlich's haematoxylin and eosin and mounted under matching No. 0 coverslips.

The HeLa cultures were exposed to " Dextraven " for varying times. The experimental results described refer to cultures treated for 4 hours, unless otherwise stated. Occasionally multiple layers of cells were found at the lower ends, with respect to the roller-tubes, of the coverslips. In later experiments these closely packed cells were removed before treatment.

EXPERIMENTAL RESULTS

HeLa cells exposed to 0·9 per cent sodium chloride solution for 4 hours were much less injured than ones treated with dextran-saline solution for the same period. Immediately after saline treatment, the cells were more transparent, owing to the solvent action of the salt solution, but decreased basophilia was not appreciable. The main difference between cultures treated with " Dextraven " and pure saline, respectively, was that in the former the chromosomes of cells in later stages of mitosis coalesced to irregular masses during treatment. Normal metaphase plates were common in the latter. Exposure to saline increased the percentage of recently degenerated cells but after 24 hours in normal medium many viable mitotic and only few pyknotic cells were seen in these cultures. After this time the saline-treated cultures behaved like untreated controls and grew normally. No delayed cytotoxic effects were seen in cultures exposed to pure saline solution. This contrasted with the changes found in dextran-saline treated cultures.

The cells of the untreated cultures, which received only standard culture medium at all times, remained normal throughout the experiments. These control cultures were necessarily sub-cultured at intervals. A typical healthy HeLa cell has basophilic cytoplam and a deeply stained nucleus with well defined structure. In treated cultures which showed reduced basophilia, the period of staining with haematoxylin was increased and that with eosin unchanged. Decreased basophilia in affected cells was therefore not due to inadequate staining.

The effects of " Dextraven " upon cells grown as a single cell layer are described below. The cytological changes observed in HeLa cells at the end of treatment for 4 hours differed in resting and dividing cells, respectively. The latter were much more severely damaged. The intact resting cells were rather more sharply defined in structure than untreated cells and appeared literally more insubstantial. They also stained less intensely with haematoxylin. Otherwise, resting cells were relatively normal in morphology although some tended to be rounded. The majority of the treated cells had the usual extended cytoplasmic processes unless in areas of confluent growth in which the epithelial habit was characteristic. Some resting cells were not intact and were partly lyzed. These, however, were not greatly in excess of the usual incidence of recently degenerated cells in normal cultures. Exposure to " Dextraven " probably accelerated the rate of degeneration of sickly cells.

In contrast with these effects on resting cells, those on some classes of dividing cells were immediately irreversibly harmful. These were cells in the division phases characterized by the absence of nuclear membranes—prometaphases to early telophases inclusive. The chromatin clumped into irregular deeply staining masses in which individual components were not always distinguishable (Fig. 1). Normal metaphase plates of typically separated chromosomes were not found. The cells thus damaged during division appeared inevitably to die. Many of the dead cells found in 24-hour-old cultures clearly represented cells originally damaged during anaphase or telophase. Other pyknotic nuclear masses with no evidence of a nuclear membrane almost certainly represented damaged prometaphases and metaphases.

A proportion of resting cells showed cytotoxic effects one day after treatment. Their nuclei were relatively lightly but diffusely stained to give an almost homogeneous appearance; the nucleoli were sometimes faintly visible. A similar appearance has been seen in chick fibrocytes exposed to iron-dextran complex. The cytoplasm of the HeLa cells was also only lightly stained with haematoxylin and the cell margins were not sharply defined. These cells were of the usual size range. Others, which were shrunken and had pyknotic nuclei, had been damaged during mitosis by direct contact with " Dextraven ".

EXPLANATION OF PLATE

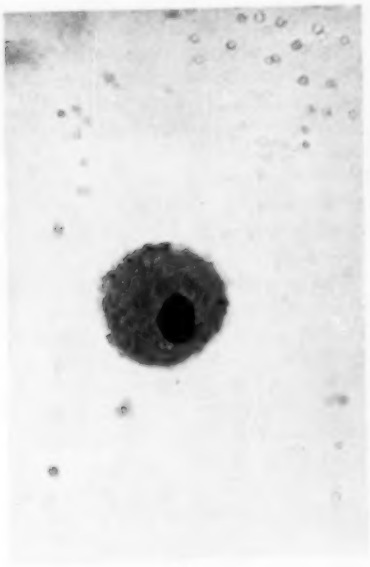
Cytopathological effects of " Dextraven " upon HeLa cells

FIG. 1.—Coalescence of metaphase chromosomes to a pyknotic mass in a cell fixed at end of treatment for 4 hours. ×320.

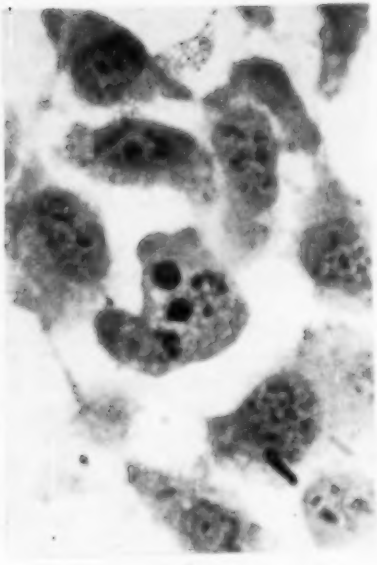
FIG. 2.—Cell showing degeneration of dividing nucleus, 7 days after treatment of culture for 4 hours. ×320.

FIG. 3.—Concurrent degeneration of resting nuclei, 12 days after treatment of culture for 4 hours. ×96.

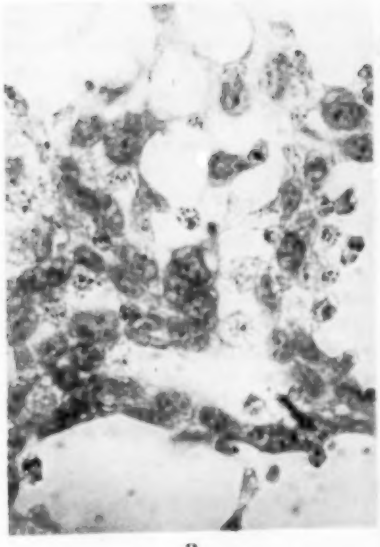
FIG. 4.—Acidophilic cells 24 hours after treatment for 8 hours. These cells contained no visible basophilic substance. ×320.



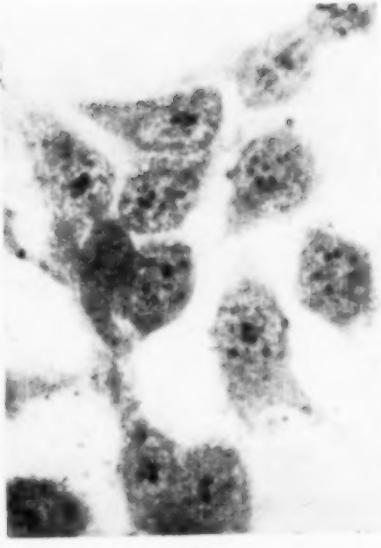
1



2



3



4

The viable resting cells were usually less basophilic than control cells. Cultures of this age contained numerous dividing cells and many of the divisions were normal. Abnormal mitoses, polymorphic and binucleate cells were seen in untreated control cultures. However, there was some evidence that " Dextraven " had inhibited cell cleavage. Unusual clumping of chromatin of cells in division at the time of fixation was not seen in cultures taken one day after treatment. This effect appeared to be due to application of " Dextraven " to cells during mitosis.

Forty-eight hours after treatment the mitotic incidence in treated and control cultures was of the same order. The majority of the treated cells were viable and few recently degenerated cells were found. By this time the treated cells had apparently recovered from the initial effects of exposure to " Dextraven ".

During the first week after treatment the cells multiplied rapidly. The denser population of the untreated control cultures was probably due rather to their uninterrupted growth than a difference in growth rates. At about the 7th day after treatment the mitotic rate began to decrease. Mitoses were still common in treated cultures but a large proportion of late prophase and especially prometaphase nuclei were abnormal (Fig. 2). In the latter class nucleoli sometimes perisisted in the degenerating cells. Cells with abnormal nuclei were unable to complete division, often became much enlarged, and finally autolyzed. Fragmentation and dissolution of chromosomes was typical but a more specific effect was incomplete individual chromosome formation. Such a chromosome appeared to be eroded, due to irregularities in the thickness and continuity of the basophilic material on the underlying thread. The latter was faintly stained where devoid of nucleic acid. A small proportion of cells appeared to have degenerated during metaphase ; in these cells normally shaped chromosomes were intermingled with denuded chromosome threads and basophilic debris. Failures of other division phases were seen but could not be definitely assigned to the effects of " Dextraven ". Many otherwise apparently normal cells were deficient in basophilic material, especially in cytoplasm, at this time.

Cultures examined two weeks after exposure to " Dextraven " were usually grossly degenerated. The comparable control cultures were healthy. The majority of the cells had degenerated. Viable survivors were commonly less basophilic than normal and few had the typical basophilic cytoplasm of healthy HeLa cells. Many of the nuclei had peculiar, sometimes refractile, densely haematoxylin-stained blebs arising from chromatin (Fig. 3). These particular nuclei were otherwise stained only faintly with haematoxylin and often in cells with purely acidophilic cytoplasm. The refractile nature of the intranuclear blebs was not a constant feature and may have been associated with incomplete removal of iodine, used to remove excess mercuric chloride after fixation, before staining. The blebs appeared to be viscous and were often seen raised above the general cell surface. Although seen free in cytoplasm of cells with lytic nuclei these droplets were derived from intranuclear material. Cells thus affected eventually autolyzed completely.

The delayed degenerative changes continued for a further week or more and were characterised by a loss of basophilic substances from nuclei and cytoplasm. This loss preceded manifest degeneration. Cultures without dense areas of cells superimposed in several layers degenerated completely during the third or fourth week after treatment with " Dextraven ". In such cultures a very few cells had survived for a long time after the deaths of other cells. These survivors often

differed greatly in appearance from the usual types of HeLa cells but may not have been associated with exposure to dextran. They were very large in area, mono- or multi-nucleate and were edged by a deeply stained, possibly reflected, zone.

When packed masses of cells were exposed to " Dextraven " for 4 hours many of the underlying cells growing in contact with the coverslips were relatively unaffected. The progeny of these cells sometimes recolonised the coverslips and overgrew the eosinophilic cell remnants.

The sequence of changes in treated cultures was initially a loss of basophilic and other substances and lethal effects on cells dividing at the time of treatment, followed by a delayed cytotoxic action resulting in gross degeneration of the cultures. Prior to the development of delayed toxicity the cell population increased visibly. The collateral cells of a single group often underwent similar degenerative changes concurrently, although the timing of these changes varied from group to group. The delayed cytotoxic effects were associated with reduced nuclear and cytoplasmic basophilia, failure of nuclear replication processes and unusual nuclear lesions. These observations together suggest the possibility that the primary injury to parent cells was related to hereditary material and intensified with successive cell divisions until the derangement became lethal. Intact nuclear membranes appeared to impede the penetration of dextran, presumably of the larger molecules present in " Dextraven ".

The effects upon HeLa cells of administration of " Dextraven " for 4 hours have been described since exposure for this period enabled most of the treated cells to withstand the acute toxicity of dextran and to multiply repeatedly until the manifestation of delayed toxic effects. Longer (6–8 hours) exposure to " Dextraven " had acute cytopathological effects essentially similar to those described. These were, however, more severe. They included a higher proportion of grossly degenerated cells ; a greater loss of basophilic material, especially from nucleoli and cytoplasm, and from mitotic spindles. In many such cultures fixed 24 hours after treatment very little substance stainable by Ehrlich's acid haematoxylin was retained by the cells. These acidophilic cells did not reproduce but viewed *in situ* in the culture vessels they looked morphologically intact (Fig. 4).

Later work has shown that, in addition to the conditions of administration, the response of HeLa cells to " Dextraven " is greatly modified by the nutritive and physiological state of the cells at the time of treatment. Actively dividing, well-fed cells were much more resistant to the toxicity of dextrans than cells taken from exhausted growth or fresh maintenance media. The original observations were made on treated starved cells. These rapidly lost their basophilic content, and presumably other substances, and endured in an apparently unchanged extracted state for an indefinite time. These deficient cells did not recover spontaneously but have been observed, if not too injured, to take up nucleic acids from suitable media. This phenomenon is being investigated.

DISCUSSION

The cytopathological effects of " Dextraven " resulted from the presence of dextran in this product. The acute initial toxicity was not remarkable in the severe experimental conditions used. But the manifestation of distinctive lethal effects, delayed until treated cells had given rise to several generations, was perhaps more unusual.

The basophilic substances lost from treated cells and their descendants included DNA and RNA. The evidence available implies that dextran-saline also leached out other cell substances. Preliminary cytochemical studies have confirmed the loss of nucleic acids. HeLa cells have been found to be more susceptible to dextran than some other types of cells, for example, Earle's L–929 strain of mouse fibroblasts. Degenerating autolysing cells often tend to lose their nucleic acids. However, the nature and time sequence of pathological changes, including loss of basophilia and nuclear lesions, strongly suggest that the delayed toxicity resulted from injury to cell structures containing nucleic acids and involved in replication processes. The initial lesions of intermitotic cells were not incompatible with survival and reproduction. HeLa cells have been found to tolerate "Dextraven" exposures less severe than those reported here. It is not impossible that small doses of dextran could lead to minute non-lethal biochemical and structural lesions involving nucleic acids. These might result in biological changes among the progeny of injured cells. The carcinogenicity of iron-dextran complex ("Imferon") established by Richmond (1957, 1959) and Haddow and Horning (1960) may be related in part to the dextran content of this preparation. HeLa cells, derived from a human carcinoma, are not wholly suitable for the investigation of the long term biological effects of dextran. Such studies, as well as others on specific cytological effects of dextran fractions, are being made on normal cells.

SUMMARY

1. The cytopathological effects of a dextran-saline preparation ("Dextraven") upon growing HeLa cells are described.

2. During exposure to "Dextraven" dividing cells in the mitotic phases lacking nuclear membranes are killed.

3. Intermitotic cells appear to recover from the initial effects of the agent but, provided they have received a minimum dosage, later die after a number of cell generations.

4. The cytopathological effects described indicate that the delayed toxicity is associated with injury to cell components containing nucleic acids and involved in replication processes.

5. It is suggested that these phenomena may be related to the known carcinogenicity of an iron-dextran complex ("Imferon").

I am indebted to Mr. G. A. Butcher for his invaluable assistance. The expenses of this work were defrayed from a block grant by the British Empire Cancer Campaign.

REFERENCES

HADDOW, A. AND HORNING, E. S.—(1960) *J. nat. Cancer Inst.*, **24**, 109.
PEREIRA, H. G. AND KELLY, B.—(1957) *J. gen. Microbiol.*, **17**, 517.
RICHMOND, H. G.—(1957) *Scottish med. J.*, **2**, 169.—(1959) *Brit. med. J.*, i, 947.

THE SULPHYDRYL LEVELS OF MOUSE SKIN DURING TREATMENT WITH POLYCYCLIC HYDROCARBONS AND CROTON OIL

G. CALCUTT AND JOAN COATES

From the Department of Cancer Research, Mount Vernon Hospital and the Radium Institute, Northwood, Middlesex

Received for publication April 28, 1961

As a consequence of previous experimental work concerning the sulphydryl (–SH) levels of target and non target tissues of various animal species subjected to a wide variety of chemical carcinogens Calcutt, Doxey and Coates (1960, 1961) have suggested that a rise in target tissue –SH levels during the tumour induction period is a prerequisite of tumour formation. Support for this viewpoint is given by Crabtree's (1944, 1945, 1946) findings that agents expected to interfere with cellular –SH are effective anticarcinogens.

The present work was designed to examine the effects of some further polycyclic hydrocarbons on sulphydryl levels in mouse skin and also to see whether croton oil in its cocarcinogenic role influenced the –SH content of the tissue to which it was applied.

The technique used for the measurement of skin –SH was identical with that used in earlier work. Full details are given by Calcutt and Doxey (1959) and Calcutt *et al.* (1960).

EXPERIMENTAL

Seventy Strong A male mice, all fifteen weeks old, were divided into five groups of fourteen each. One group were untreated and served as controls for the remaining four groups. These were treated as below :

Group I. Painted twice weekly with 0·2 ml. of 0·1 per cent anthracene.

Group II. Painted twice weekly with 0·2 ml. of 0·1 per cent pyrene.

Group III. Painted twice weekly with 0·2 ml. of 0·1 per cent 1,2 : 5,6-dibenzanthracene.

Group IV. Painted twice weekly with 0·2 ml. of 0·1 per cent 1,2-benzanthracene.

All hydrocarbons were applied as solutions in acetone, and the paintings in each case were of an area of approximately 1 cm. square on the centre of the back.

At daily intervals for four days and then at approximately weekly intervals up to ten weeks one control animal and one from each experimental group were killed. A portion of treated skin was taken, or a comparable piece in the case of the control animals, and the –SH level was estimated.

In the case of the control animals only minor fluctuations were detected in –SH levels, and so a mean figure and a standard deviation have been calculated. The results for Group I (anthracene) are shown in Fig. 1. It will be seen that there

is a persistent slight lowering of –SH levels as compared with the control value. In the case of Group II (pyrene) the results are displayed as Fig. 2. Here there would appear to be little or no effect on –SH levels, as the experimental figures

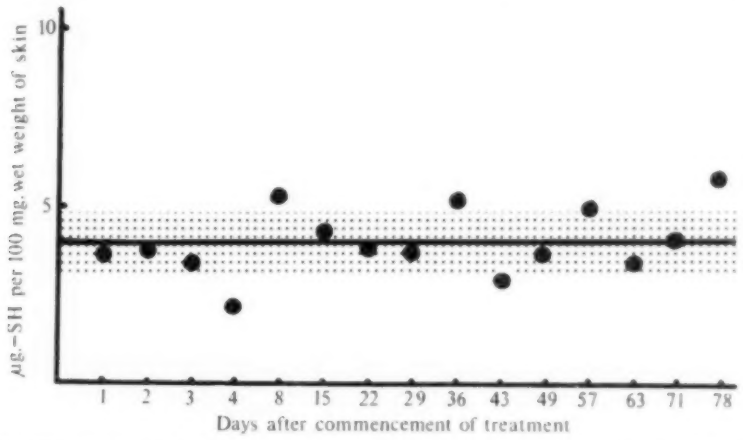


FIG. 1.—The effects of repeated applications of anthracene on mouse skin –SH levels. The mean control figure is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

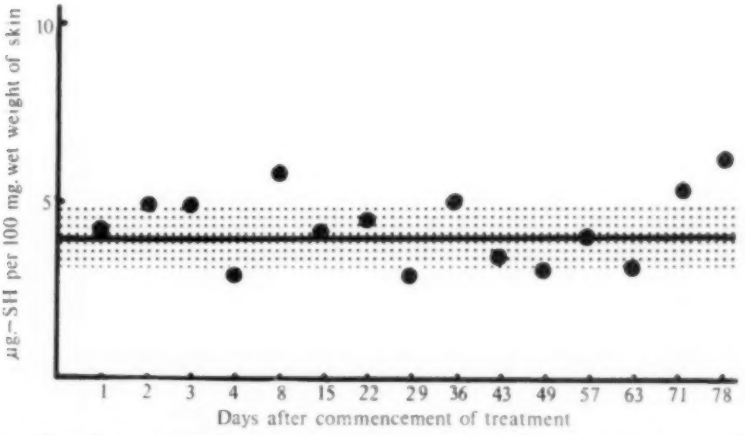


FIG. 2.—The effects of repeated applications of pyrene on mouse skin –SH levels. The mean control figure is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

adhere closely to the mean value. Group III (1,2 : 5,6-dibenzanthracene) differs from the two previous groups in that they represented non-carcinogenic hydrocarbons but the present one is a moderately potent carcinogen to mouse skin. The experimental findings, shown in Fig. 3, indicate a different state of affairs.

This time the –SH levels rise above the mean level and tend to remain elevated throughout the experiment. In Group IV (1,2-benzanthracene) the agent is only

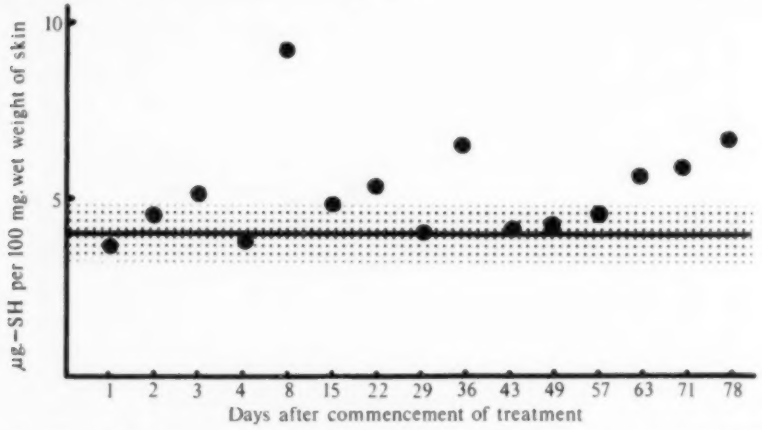


FIG. 3.—The effects of repeated applications of 1,2 : 5,6-dibenzanthracene on mouse skin –SH levels. The mean control figures is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

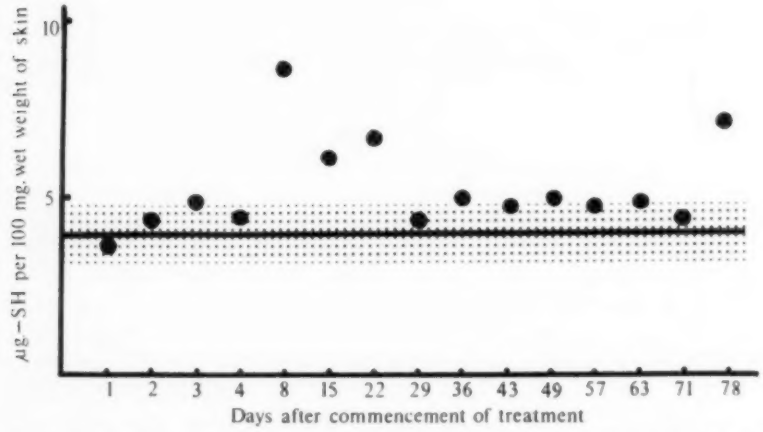


FIG. 4.—The effects of repeated applications of 1,2-benzanthracene on mouse skin –SH levels. The mean control figure is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

a very weak carcinogen but the results, shown in Fig. 4, indicate that a well defined rise in skin–SH levels was obtained.

A second batch of seventy-five Strong A male mice aged fourteen weeks were divided into five groups of fifteen each. One group were untreated and acted

as controls, whilst the others were treated as below :

Group A. Painted once only with 0·2 ml. of 0·1 per cent 7,12-dimethylbenzanthracene.

Group B. Painted twice weekly with 0·2 ml. of 0·1 per cent 7,12-dimethylbenzanthracene.

Group C. Painted once only with 0·2 ml. of 0·1 per cent 7,12-dimethylbenzanthracene and then twice weekly with 0·2 ml. of 1·0 per cent croton oil.

Group D. Painted twice weekly with 0·2 ml. of 1·0 per cent croton oil.

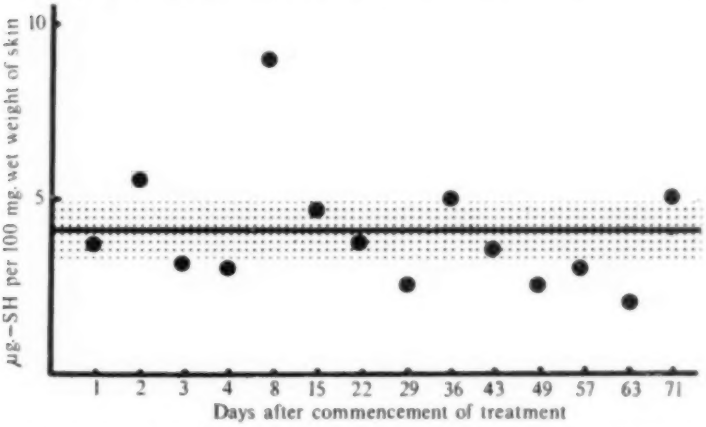


FIG. 5.—The effects of a single application of 7,12-dimethylbenzanthracene on mouse skin –SH levels. The mean control figure is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

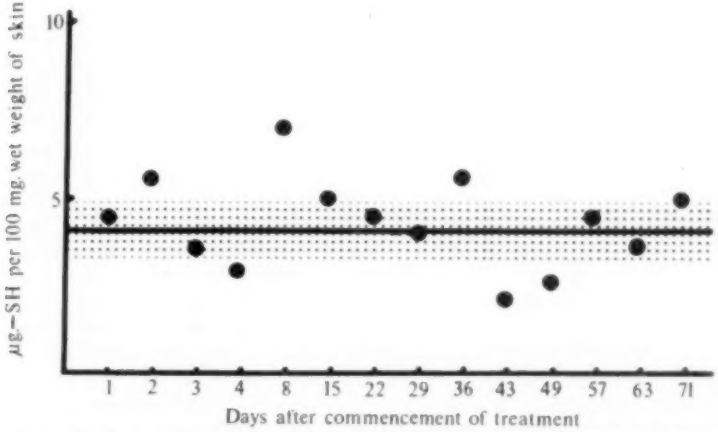


FIG. 6.—The effects of repeated applications of 7,12-dimethylbenzanthracene on mouse skin –SH levels. The mean control figure is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

As previously the hydrocarbon was applied as an acetone solution to approximately 1 cm. square of the centre of the back. Croton oil was used as an acetone

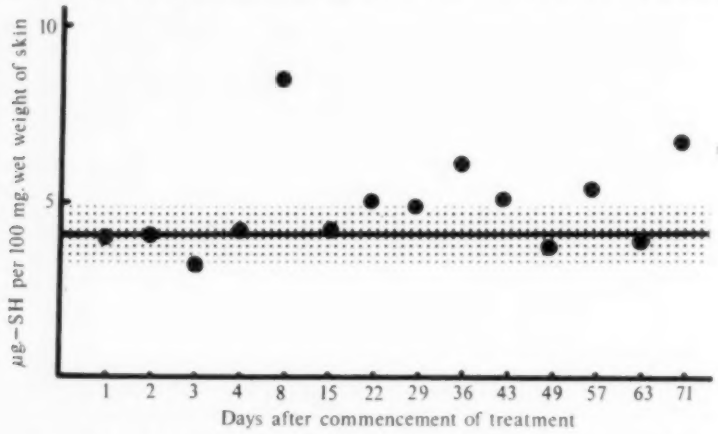


FIG. 7.—The effects of a single application of 7,12-dimethylbenzanthracene and repeated applications of croton oil on mouse skin –SH levels. The mean control figure is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

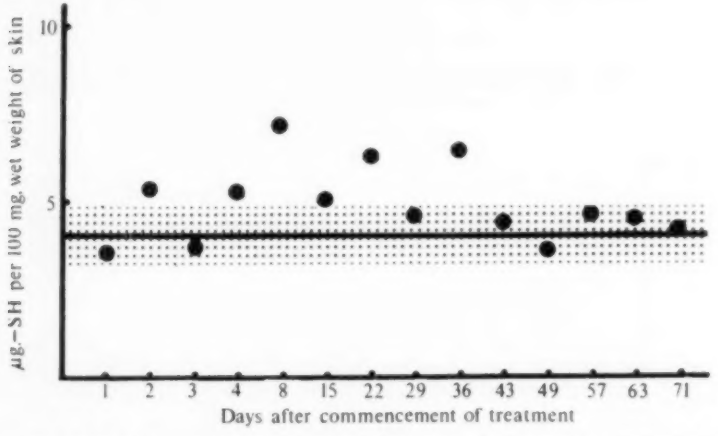


FIG. 8.—The effects of repeated applications of croton oil on mouse skin –SH levels. The mean control figure is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

solution and was applied to a slightly larger area, so that in Group C the margins of the initially treated area were covered by the later applications.

Daily for four days and then at approximately weekly intervals for ten weeks one control animal and one from each experimental Group were killed. A

portion of treated skin, or a comparable piece from the control animal, was taken from each animal for –SH estimations.

As no pattern was discernible in the control figures a mean figure and standard deviation were calculated. The results for Group A (single painting with dimethylbenzanthracene) are shown in Fig. 5. An initial slight rise is followed by a return to normal values. Group B (repeated painting with dimethylbenzanthracene) shows a rise in skin –SH values for about five weeks and then a return to normal values. These results are shown in Fig. 6. In Group C (one treatment with dimethylbenzanthracene followed by croton oil) the results—Fig. 7—fall into a different pattern. Here there is a rise in skin –SH levels after about three weeks treatment and this then persists throughout the experiment. Group D (repeated painting with croton oil) showed yet another picture. This time there was a rise in skin –SH over the period 2 days to 5 weeks. This was followed by a return to normal levels. These figures are shown in Fig. 8.

The two groups of experiments recorded above were run concurrently and it is noticeable that all estimations performed on the eighth day show very high –SH levels. No explanation has been found for this rather unusual occurrence.

DISCUSSION

The present results are in good agreement with those previously obtained by Calcutt *et al.* (1960, 1961). The carcinogenic agents, 1,2 : 5,6-dibenzanthracene and 7,12-dimethylbenzanthracene have been found to cause an elevation in mouse skin –SH levels for some period during the tumour induction phase. Additionally, the very weak carcinogen, 1,2-benzanthracene and the non-carcinogenic croton oil have been found to cause similar elevations of –SH level. The two non-carcinogens, anthracene and pyrene did not cause any rise in skin –SH levels.

On the basis of the previously suggested requirement for an elevation of target tissue –SH as a prerequisite for tumour induction the new results fall into place. After a single application of the potent carcinogen, 7,12-dimethylbenzanthracene, there is only a transient rise in –SH and a return to normal values (Fig. 5). This treatment would not be expected to give any appreciable tumour yield, but when further hydrocarbon applications are made so as to bring about conditions expected to give rise to a good tumour yield there is an –SH rise (Fig. 6). Again, if after an initial hydrocarbon application croton oil is used as a promoting agent a period of elevated –SH levels ensues (Fig. 7). Thus, the final picture is the same whether repeated hydrocarbon applications or a single application followed by croton oil is used. So we have the two different conditions which end in similar biological results showing the same biochemical effect during the period immediately preceding the visible biological response.

A comparison of Fig. 7 and 8 shows that the appearance of the elevated –SH level in the skin is delayed in the case of the hydrocarbon treated skins as compared with those treated solely with croton oil. Whether this implies tissue damage affecting a later response to the croton oil remains to be determined.

Data so far available of the mouse skin –SH response to carcinogens also suggests that the more powerful agents 7,12-dimethylbenzanthracene and 3,4-benzopyrene (Calcutt *et al.*, 1960) show a rapid and rather short lived response. The weaker agents, 1,2 : 5,6-dibenzanthracene, 1,2 : 5,6-dibenzacridine (Calcutt *et al.*, 1961) and 1,2-benzanthracene show a much more persistent response.

The possibility of a relationship between the extent and duration of the response and the latent period for tumour induction will be considered in a future publication.

In the present series of experiments anthracene has been found to depress skin –SH levels. Crabtree (1946) using 0·7 per cent solutions of anthracene as against our 0·1 per cent concentration found slight falls in skin glutathione levels up to four hours after treatment. In the same paper it was reported that anthrcene was a weak inhibitor of skin tumour induction by benzopyrene or dibenzanthracene. If the effects of anthracene and benzopyrene or dibenzanthracene when applied alternately to skin are additive then the final result would be expected to be a reduced response in the skin –SH level. Further experiments to confirm this point are in progress.

The fact that the very weak carcinogen, 1,2-benzanthracene induces a rise in skin –SH (Fig. 4) suggests that this agent could be a better promoting agent than initiating agent. In actual practice, when tested by Hadler, Darchun and Lee (1959) no promoting action whatever was detected. Since these authors used Swiss mice, a different strain to that used in the current work, and we have no information on the effects of benzanthracene on the –SH levels in Swiss mice skin, no further comment is feasible at the moment.

SUMMARY

1. Twice weekly paintings of mouse skin with 0·1 per cent solutions of 1,2 : 5,6-dibenzanthracene, 1,2-benzanthracene or 7,12-dimethylbenzanthracene cause rises in skin –SH levels.

2. Twice weekly painting of mouse skin with 0·1 per cent anthracene causes a slight lowering of skin –SH levels.

3. Twice weekly painting of mouse skin with 0·1 per cent pyrene has no effect on skin –SH levels.

4. A single painting with 0·1 per cent 7,12–dimethylbenzanthracene causes a transient rise in –SH levels followed by a return to normal levels.

5. Twice weekly painting of mouse skin with 1·0 per cent croton oil, either with or without a pretreatment with 7,12-dimethylbenzanthracene causes an elevation of skin –SH levels.

6. These results are discussed in relation to the view that a rise in tissue –SH levels is an essential prerequisite of tumour formation.

REFERENCES

CALCUTT, G. AND DOXEY, D.—(1959) *Exp. Cell. Res.*, **17**, 542.
Iidem AND COATES, JOAN.—(1960) *Brit. J. Cancer*, **14**, 746.—(1961) *Ibid.*, **15**, 149.
CRABTREE, H. G.—(1944) *Cancer Res.*, **4**, 688.—(1945) *Ibid.*, **5**, 346.—(1946) *Ibid.*, **6**, 553.
HADLER, H. I., DARCHUN, VIOLET AND LEE, KATHERINE.—(1959) *J. nat. Cancer Inst.*, **23**, 1383.

TRYPTOPHAN-NICOTINIC ACID METABOLISM IN PATIENTS WITH TUMOURS OF THE BLADDER. CHANGES IN THE EXCRETORY PRODUCTS AFTER TREATMENT WITH NICOTINAMIDE AND VITAMIN B_6

E. QUAGLIARIELLO, F. TANCREDI, L. FEDELE AND C. SACCONE

From the Institute of Biological Chemistry, University of Naples, and Institute of Biological Chemistry, University of Camerino, Italy

Received for publication March 15, 1961

CHROMATOGRAPHY was used to estimate the excretion of metabolites of tryptophan. Analysis and identification, using both paper and talc columns, has shown that, especially in pathological conditions in man and experimental animals, there are metabolic blocks at different levels in the complex series of reactions in the conversion of the tryptophan to nicotinic acid. These blocks result in the accumulation in the urine of certain metabolites. Some vitamins take part in the conversion of tryptophan into nicotinic acid and thus form examples of catalysts which play an essential part in the biological syntheses of other catalysts. Thus in the absence or deficiency of a vitamin essential for a reaction such as the conversion of tryptophan into nicotinic acid a metabolic block will appear.

Recently Auricchio, Quagliariello, Rubino and Vecchione (1959) have examined the urine of children with different diseases (leukaemia, Hodgkin's disease, hepatitis, and rickets) and have commented on the presence of abnormal metabolites of the tryptophan-nicotinic acid group. They could not, however, prove a definite relationship between the type of disease and the abnormal urine.

We are interested in the relationship between tumours of the bladder and the metabolic by-products from tryptophan degradation and in our preceding work (Quagliariello, Auricchio, Casale and Tancredi, 1958; Fedele, Trancredi and Riccardi, 1959, 1961) we have discussed the possible aetiology of cancer in relation to metabolites of tryptophan.

Bonser, Clayson, Jull and Pirah (1952) have shown that some *ortho*-aminophenols produce cancer of the urinary bladder. Allen, Boyland, Dukes, Horning and Watson (1957) showed that the two *ortho*-aminophenols, 3-hydroxykynurenine and 3-hydroxyanthranilic acid, induced bladder cancer under the same conditions. Higher concentrations of 3-hydroxyanthranilic acid and kynurenine have been found in the urine of patients with cancer of the bladder when compared with urine of either normal subjects or of those with cancer localized in organs other than the bladder (Boyland and Williams, 1955). Perhaps this indicates that a block at the level of the 3-hydroxyanthranilic oxidase (with subsequent accumulation of 3-hydroxyanthranilic acid and of 3-hydroxykynurenine) is the basis of tumours of the bladder.

Dunning, Curtis and Mann (1956) in a study of the relationship between diet and production of cancer by 2-acetylaminofluorene found that cancer of the

bladder occurred in almost all of the rats (strain Fisher 344) fed with a diet containing tryptophan (1·4–4·3 per cent). The large amounts of tryptophan necessary for the development of cancer suggest that two *ortho*-aminophenolic derivatives with carcinogenic activity, 3-hydroxyanthranilic acid and 3-hydroxykynurenine might be excreted. If 2-acetylaminofluorene caused a biochemical block in the transformation of these two compounds, it would explain the carcinogenic action in the bladder of this compound. Quagliariello and his colleagues (1958) have concluded that rats treated with 2-acetylamino-fluorene have a block at the level of 3-hydroxyanthranilic oxidase in the liver. In the present work the modification of the tryptophan-nicotinic acid metabolism by treatment with nicotinamide and vitamin B_6 in people with tumours of the bladder has been investigated.

EXPERIMENTAL PROCEDURE

Twenty-four hour specimens of urine were acidified with acetic acid (1 : 1) and centrifuged. Charcoal deactivated with 4 per cent by weight of stearic acid was added to the supernatant and the mixture shaken occasionally for about 5 minutes. The charcoal was filtered off on a small Buchner funnel and washed with water to remove the remaining salts, urea, sugars and aliphatic amino acids. It was then eluted with phenol solution and water. The eluate was evaporated on a water-bath under reduced pressure. To ensure complete removal of the phenol more water was added and the mixture again concentrated. The concentrate thus obtained was applied directly to paper chromatograms.

For routine use about 10 to 20 ml. of urine was suitable, but there was no difficulty in using as little as 5 ml. The amounts of charcoal and phenol used varied because of variations in the metabolite content of the urine, but a suitable amount could easily be estimated after some experience had been gained. If sufficient charcoal had been used the urine filtrate after the initial adsorption was almost or quite colourless.

For routine examinations one-dimensional descending chromatograms developed with butanol : acetic acid : water (4 : 1 : 5, v/v/v) were most suitable. Chromatograms run under such conditions are adequate for almost all purposes provided reference standards are run simultaneously. R_F values can be modified amongst other things, by a metabolite being displaced by other substances in a crowded region of the chromatogram, and by variations in the pH of the applied solution.

Fluorescence under ultra-violet light.—When examined in ultra-violet light which has passed through a Wood's glass filter many metabolites are revealed as characteristic fluorescent spots. Ultra-violet light is also the most convenient way of revealing the solvent front. Normal urine contains traces of many unidentified fluorescent substances, some of which may be of plant origin (from the diet) rather than true metabolites.

Ninhydrin.—Ninhydrin is of value chiefly for the aromatic amino acids and also, using a modified reagent and the resulting ultra-violet fluorescence, for tryptamine derivatives.

Ehrlich's reagent.—This is of particular value for the detection of indole derivatives, pyrrole derivatives and aromatic amines. It is convenient to have two reagents available, differing in acid strength, which will be referred to as the " normal " and " strong " Ehrlich's reagent respectively. The " normal " reagent

TABLE I.—*Chromatographic Evidence of Tryptophan Metabolites in Urine of Patients with Cancer of the Bladder*

Case number	Kynurenine	3-hydroxy-kynurenine	3-hydroxy-anthranilic acid	Anthranilic acid
1. D. E.	+++	+	+++	−
2. A. L.	++	−	++	+
3. G. S.	+++	−	++	+
4. R. A.	−	−	+	+
5. R. G.	++	−	++	+
6. C. A.	++	−	+	−
7. D. M.	++	−	++	+
8. C. E.	−	−	++	−
9. Q. F.	+	−	++	+
10. C. S.	−	−	+	−
11. T. P.	−	−	+	+
12. T. C.	+	−	−	+
13. P. L.	+	−	−	−

+++ = constant presence and large quantities; ++ = constant presence and small quantities; + = not always present or only in small quantities; − = never present.

TABLE II.—*Chromatographic Evidence of Tryptophan Metabolites in Urine of Patients with Cancer of the Bladder, Before and After Treatment with Nicotinamide* (400 *mg.*/pro die *for* 15 *days*)

	Before nicotinamide treatment				After 15 days of nicotinamide treatment			
Case number	Kynurenine	3-hydroxy-kynurenine	3-hydroxy-anthranilic acid	Anthranilic acid	Kynurenine	3-hydroxy-kynurenine	3-hydroxy-anthranilic acid	Anthranilic acid
14. T. M.	+	−	+++	++	+	−	−	−
15. M. G. G.	++	−	+++	−	−	−	−	−
16. C. A.	++	−	+++	+	+	−	−	−
17. B. F.	++	−	+++	+	+	−	+	+
18. C. D.	++	−	++	+	++	−	−	−
19. A. S.	+	−	++	−	−	−	−	−
20. D. C.	−	−	++	+	−	−	++	+
21. B. P.	−	−	+++	+	−	−	+	+
22. T. V.	−	−	+++	++	−	−	−	−
23. G. M.	+	−	+++	+	+	−	−	+
24. L. S.	++	−	++	−	+	−	−	−
25. P. L.	+++	−	+++	+	−	−	−	−
26. G. R.	++	−	++	+	++	−	++	−
27. D. D. E.	−	−	+++	−	−	−	−	−
28. A. L.	−	−	+	+	−	−	+	++
29. G. S.	−	−	++	+	−	−	++	++
30. G. G.	+++	−	++	−	++	−	++	−
31. D'A. M.	+	−	+++	+	+	−	−	−
32. S. E.	+++	−	++	−	++	−	−	−
33. C. M.	−	−	+	+	−	−	+	+
34. C. D.	++	−	+++	+	−	−	−	−
35. B. S.	+	−	++	−	+	−	−	−
36. P. V.	+++	−	++	+	++	−	++	++

+++ = constant presence and large quantities; ++ = constant presence and small quantities; + = not always present or only in small quantities; − = never present.

TABLE III.—*Chromatographic Evidence of Tryptophan Metabolites in the Urine of Patients with Cancer of the Bladder, Before and After Treatment with Vitamin B_6 (400 mg./pro die for 15 days)*

Case number	Before vitamin B_6 treatment				After 15 days of vitamin B_6 treatment			
	Kynurenine	3-hydroxykynurenine	3-hydroxyanthranilic acid	Anthranilic acid	Kynurenine	3-hydroxykynurenine	3-hydroxyanthranilic acid	Anthranilic acid
37. E. A.	+++	+++	+	−	−	++	+	−
38. D. G.	−	++	+	+	−	−	−	−
39. N. L.	+	++	+	−	+	+	+	−
40. D. S.	+	+++	−	−	+	++	−	−

+++ = constant presence and large quantities; ++ = constant presence and small quantities; + = not always present or only in small quantities; − = never present.

TABLE IV.—*Chromatographic Evidence of Tryptophan Metabolites in the Urine of Patients with Cancer of the Bladder, Before and After Treatment with Nicotinamide and vitamin B_6 (200 mg. + 200 mg./pro die for 15 days)*

Case number	Before vitamin B_6 treatment				After 15 days of nicotinamide and vitamin B_6 treatment			
	Kynurenine	3-hydroxykynurenine	3-hydroxyanthranilic acid	Anthranilic acid	Kynurenine	3-hydroxykynurenine	3-hydroxyanthranilic acid	Anthranilic acid
41. P. M.	++	++	+++	+	++	+	++	−
42. C. M.	+++	−	+++	−	+	−	+	−
43. S. A.	++	+	+++	++	+	−	−	+
44. A. G.	++	++	++	+	+	+	+	−
45. D'A. V.	+	+	+	+	−	−	−	−
46. D. G.	+	++	+++	+	++	−	+	−
47. S. M.	+	+	+	++	+	−	−	−

+++ = constant presence and large quantities; ++ = constant presence and small quantities; + = not always present or only in small quantities; − = never present.

is a 2 per cent (w/v) solution of *p*-dimethylaminobenzaldehyde in approximately 1.3N–HCl. The "strong" reagent is a 2 per cent (w/v) solution of the aldehyde in approximately 6 N–HCl. After spraying the papers are allowed to dry at room temperature. With the "normal" reagent, aromatic amines give a yellow colour immediately, whereas colours from indole derivatives may take some time to form. At the pH of the "strong" reagent derivatives of aromatic amines are formed but remain colourless, whereas indole derivatives including indican, give a colour rapidly. It is thus possible to distinguish the two types of compounds even if present in the same spot. Ultimately the picture obtained with both reagents is the same.

Ammoniacal silver nitrate.—The papers are sprayed and allowed to dry at room temperature. The reagent is of special value in detecting substances containing readily oxidizable groups such as *o*-aminophenol or catechol derivatives. These rapidly give black or brown-black spots. Many other substances will react, but more slowly.

Urine from 47 patients with cancer of the bladder was collected and analysed every other day for 20 days. At the end of this time 23 of these patients were given 400 mg. per day of nicotinamide (Nicotinamide " Lepetit ") by parenteral injection for 15 days. Four other patients were injected with 400 mg. Vitamin B_6 (Benadon " Roche ") per day for 15 days. Seven patients were treated with both vitamin B_6 (200 mg.) and nicotinamide (200 mg.). In some patients the studies were continued after surgical removal of the tumours.

RESULTS

In the urine from 44 of the patients 3-hydroxyanthranilic acid was present ; kynurenine and anthranilic acid were present in 35 and 31 of these cases respectively. 3-Hydroxykynurenine was found in only 11 patients. Additional metabolites such as acetylkynurenine, anthranilic acid glucuronide, kynurenic acid and xanthurenic acid were occasionally present.

In 3 patients 3-hydroxyanthranilic acid was never detected but only kynurenine and some of its derivatives.

In 14 of the 23 patients treated with nicotinamide there was a gradual disappearance of the 3-hydroxyanthranilic acid often with a complete change in the chromatographic pattern. In 2 patients the nicotinamide treatment, begun only after surgical operation, caused the excretion of the metabolites of the tryptophan-nicotinic acid group to return to normal.

Vitamin B_6 was given to four patients whose urine contained large amounts of 3-hydroxykynurenine. Normal functioning of the excretory system was obtained only in one case. Another 7 patients were treated with both nicotinamide and vitamine B_6, but normal excretion was obtained in only 3 cases, probably because only a half-dose of vitamin B_6 and nicotinamide was administered.

(1) Of the three patients who never excreted 3-hydroxyanthranilic acid, biopsies showed that two did not have cancer of the bladder.

(2) All 47 of the cases investigated were followed for 20 days. In some patients the presence of 3-hydroxyanthranilic acid in the urine was always detectable ; in others the excretion of this metabolite was not constant.

(3) In recent studies by others (Benassi and Perissinotto, 1959, 1960) 3-hydroxyanthranilic acid has not been found, although a large number of cases were examined.

(4) In 13 of the subjects examined we have followed the urinary excretion of the metabolites of tryptophan even after surgical removal of the tumour and we have not found that the excretory products have returned to normal. This is important, because it indicates a dangerous and permanent metabolic block.

(5) The nicotinamide treatment appears to cause normal metabolism of tryptophan. This treatment was suggested by our previous researches which indicated the probable role of DPN in the 3-hydroxyanthranilic acid oxidation.

DISCUSSION

From our results it is evident that in different diseased conditions the normal mechanisms for degradation of tryptophan are altered and that some metabolites of the tryptophan-nicotinic acid group accumulate in the urine. However, with paper chromatography it is not always possible to demonstrate a pattern which

differs according to the disease. We feel, however, that there is a characteristic change in the excretory products of people afflicted with cancer of the bladder.

In agreement with our earlier studies (Fedele, *et al.*, 1959, 1961 ; Quagliariello *et al.*, 1958) the researches of Boyland and Williams (1955), and experiments of Tompsett (1959), the results show that 3-hydroxyanthranilic acid is usually present in the urine along with other metabolites of the tryptophan-nicotinic acid group —kynurenine and anthranilic acid—in bladder cancer patients.

After our studies and those of others we have cited, we cannot consider research regarding the correlation between blocks in the tryptophan nicotinic acid metabolism and cancer of the bladder as finished. However, it appears more evident that there is a relationship between metabolites of the tryptophan and cancer of the bladder.

SUMMARY

Urine from patients with bladder cancer usually contains 3-hydroxyanthranilic acid, kynurenine and anthranilic acid. The excretion of 3-hydroxyanthranilic acid is reduced by treatment with large doses of nicotinamide.

REFERENCES

Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S. and Watson, J. G.—(1957) *Brit. J. Cancer*, **11**, 212.

Auricchio, S., Quagliariello, E., Rubino, A. and Vecchione, L.—(1959) *Bioch. applic.*, **3**, 113.

Benassi, C. A. and Perissinotto, B.—(1959) ' Res. V Jornadas Bioquimicas Latinas,' Barcelona (Mayo), p. 84.—(1960) *Farmaco*, **15**, 323.

Bonser, G., Clayson, D. B., Jull, I. W. and Pirah, L. N.—(1952) *Brit. J. Cancer*, **6**, 412.

Boyland, E. and Williams, D. C.—(1955) *Biochem. J.*, **60**, V.

Dunning, W. F., Curtis, M. R. and Mann, B. D.—(1956) *Cancer Res.*, **10**, 454.

Fedele, L., Tancredi, F. and Riccardi, I.—(1959) *Atti Soc. it. Urol.*, **32**, (in press).—(1961) *Minerva urol.*, **13**, 1.

Quagliariello, E., Auricchio, S., Casale, M. and Tancredi, F.—(1958) *Boll. Soc. ital. Biol. sper.*, **34**, 970.

Tompsett, S. C.—(1959) *Clin. chim. Acta*, **4**, 411.

STUDIES ON ISOLATED TUMOUR MITOCHONDRIA: OXIDATIVE PHOSPHORYLATION AND FATTY ACID OXIDATION BY RAT HEPATOMA MITOCHONDRIA

P. EMMELOT AND C. J. BOS

From the Department of Biochemistry, Antoni van Leeuwenhoek-Huis: The Netherlands Cancer Institute, Amsterdam, The Netherlands

Received for publication March 22, 1961

In a previous paper from this laboratory (Emmelot, Bos, Brombacher and Hampe, 1959) an extensive survey was presented of the metabolic properties of hepatoma mitochondria with special reference to the transplanted rat hepatoma BY 225. This hepatoma was of the solid type and, by lack of evidence to the opposite, was considered to be suitable for a metabolic comparison with the normal rat liver. Meanwhile three other transplanted rat hepatomas have been established, bearing the case numbers BY 446, BY 448 and BY 463. The question of the cell origin of the four rat hepatomas has been discussed in the preceding paper (Emmelot, Hampe, Bos and Reyers, 1961) on account of the levels of deoxycytidylate deaminase activity, an enzyme which was considered by Pitot and Potter (1960) to be a likely parameter for distinguishing between parenchymal and bile duct (tumour) cells. In the present investigation we have been concerned with some metabolic properties, relating to adenosine triphosphate (ATP) production of the mitochondria from the three newly-established hepatomas. These experiments were carried out with a dual purpose in mind. It has been shown repeatedly (Emmelot *et al.*, 1959) that mitochondria from different tumours may behave non-uniformly and, accordingly, it was of interest to investigate, first, to what extent the metabolic properties of mitochondria from transplanted rat hepatomas might vary and, secondly, how far the latter data might furnish information that would allow a classification of the rat hepatomas. Non-uniformity in enzymic behaviour between the hepatoma mitochondria was indeed encountered in the present experiments, one of the hepatomas, BY 448, yielding mitochondria which, in contrast to the other hepatoma mitochondria, were deficient in oxidative phosphorylation and fatty acid oxidation. In view of the fact that of the four rat hepatomas studied, the latter showed consistently the lowest content of deoxycytidylate deaminase activity (Emmelot *et al.*, 1961), we have made some effort to find the reason underlying the difference in mitochondrial activities observed between the hepatomas. Pertinent to this question was the earlier finding (Emmelot *et al.*, 1959) that less satisfactory results were obtained with mitochondria from BY 225 hepatoma transplants which had grown to full size and contained necrosis (the latter was removed: "old" transplants) than with mitochondria from smaller "young" transplants. Accordingly, in the present experiments attention was also directed to the possible effect of transplant size and "quality" on mitochondrial activity. For, if the transplants of the BY 448 hepatomas were to contain

more necrosis than those of the other hepatomas or if only " old " transplants of the BY 448 hepatoma had been used, the difference in the mitochondrial activity would have to be ascribed to this artefact. This not being the case, we tried to find some other explanation for the abnormal behaviour of the BY 448 hepatoma mitochondria. In this we were led by the results of other experiments which had shown that the complete removal of the fat droplets from a tumour homogenate was essential in order to obtain mitochondria with a relatively low " free " ATPase activity and a demonstrable DNP-activated ATPase (Emmelot *et al.*, 1959). A second lead was provided by the finding that the *in situ* fine structure of the major part of the mitochondria in liver cells, after the *in vivo* administration of an acutely toxic dose of the hepatocarcinogen dimethylnitrosamine, was not changed (Emmelot and Benedetti, 1960), whereas upon isolation of the particles from such livers an increased lability of their structure and function was noted (Emmelot, Bos, Brombacher and Reyers, 1960 ; Emmelot, Bos and Reyers, 1960). Thus it was considered likely that the mitochondria suffered damage during the homogenisation of the tissue and the subsequent isolation procedure. Since non-structurally-bound fat did accumulate in these livers to a marked extent (Emmelot and Benedetti, 1960) and since fats may damage mitochondria (Pressman and Lardy, 1956 ; Avi-Dor, 1960), the top fatty layer from the homogenates of the treated livers was separated and, after washing, added to normal rat liver mitochondria in order to see whether the properties of the latter might be changed. This fat produced an activation of the latent, Mg^{2+}-dependent, ATPase and an inhibition of the DNP-activated ATPase of fresh normal liver mitochondria (Emmelot, Bos, and Reyers, 1960). Similar experiments were, therefore, carried out in the present investigation with the fat obtained from rat liver and the various hepatoma homogenates.

MATERIALS AND METHODS

The tumours studied in the present investigation have been described in the preceding paper (Emmelot *et al.*, 1961). Transplants, labelled as " young ", were harvested after growth during periods shorter than or equal to those mentioned in the latter paper. Such transplants contained only occasionally necrosis and were then designated as " old transplants ". Otherwise, transplants labelled " old", had been grown for longer periods than those indicated. Necrotic areas, when present, were always removed before homogenisation of the tissues. In view of the fact that the transplants were maintained intraperitoneally, the utmost care was taken to remove the adhering fat as completely as possible. The isolation of the mitochondria (0·25 M sucrose containing 0·001 M ethylenediamine tetra-acetate (EDTA) and the measurements of oxidative phosphorylation, fatty acid oxidation and ATPase activity were carried out as described previously (Emmelot *et al.*, 1959). Non-structurally-bound lipids were separated from the hepatoma homogenates as indicated in the text.

RESULTS

Oxidative phosphorylation

Table I contains typical data illustrating the oxidative and phosphorylative activities of mitochondria isolated from " young " transplants of the three rat hepatomas BY 446, BY 463 and BY 448 in the presence of glutamate, diphos-

phopyridine nucleotide (DPN), cytochrome *c*, and the " high energy " phosphate trapping system glucose-hexokinase. Incubation was carried out at pH 7·0 and 7·4 in histidine buffer at 27° C. (Emmelot, Bos, Brombacher and Hampe, 1959). Oxidative phosphorylation by the hepatoma mitochondria, like that by liver mitochondria, was somewhat more efficient at the former than at the latter pH. Earlier experiments had shown that the Mg^{2+}-activated ATPase of another transplanted rat hepatoma (BY 227) was less active at pH 7·0 than at 7·4. The P : O ratios obtained with the mitochondria from the hepatomas BY 446 and 463 were satisfactory and, at pH 7·0, equalled that obtained with normal liver mitochondria. However, the efficiency of the oxidative phosphorylation of the BY 448 hepatoma mitochondria was very low. In this respect the latter mitochondria behaved quite differently from the BY 446, BY 463, and the BY 225 mitochondria studied earlier (Emmelot *et al.*, 1959). The specific activity of the mitochondrial

TABLE I.—*Oxidation and Phosphorylation by Mitochondria from " Young " Transplants of the Rat Hepatomas BY* 446, *BY* 448 *and BY* 463, *in the Presence of Glutamate*

Mitochondria were isolated in 0·25 M sucrose containing 0·001 M EDTA (pH 7·4) and incubated (DPN and glucose-hexokinase being added) as previously described (Emmelot *et al.*, 1959).

Tumour	Transplant generation	Number of transplants used	pH of incubation medium	Mitochondrial nitrogen per flask (mg.)	Time of incubation (minutes)	Oxygen consumption (μatoms)	Phosphate uptake (μmoles)	P : O	Oxygen consumed/mg. mitochondrial N/hour (μatoms)
BY 446	13	1	7·4	0·80	15	7·6	12·0	1·6	38·0
	15	2	7·0	0·76	15	6·3	14·9	2·4	33·6
BY 463	2	1	7·4	1·25	10	7·0	13·3	1·9	33·6
	3	2	7·0	1·01	10	6·1	15·3	2·5	36·6
BY 448	13	1	7·4	0·74	15	5·1	2·3	0·4	27·2
	14	2	7·0	0·73	15	5·0	3·5	0·7	26·7

TABLE II.—*Oxidation and Phosphorylation by Mitochondria from " Old " Transplants of the Rat Hepatomas* BY 446, BY 448 *and* BY 463, *in the Presence of Glutamate.*

Tumour	Transplant generation	Number of Transplants used	pH of incubation medium	Mitochondrial nitrogen (mg. flask)	Time of incubation (minutes)	Oxygen consumption (μatoms)	Phosphate uptake (μmoles)	P : O	Oxygen consumed/mg. mitochondrial N/hour (μatoms)
BY 446	12	1	7·0	1·02	20	3·8	0·3	0·2*	11·2
			7·4	1·02	20	5·0	0·0	0*	15·0
BY 463	5	1	7·0	1·39	15	5·4	8·2	1·5	15·5
BY 448	12	1	7·0	1·05	15	7·9	6·4	0·8	31·0

* In the absence of glucose-hexokinase

glutamate oxidation*, expressed as microatoms oxygen consumed/mg. mitochondrial nitrogen/hour, was of the same order of magnitude for all the hepatomas, that of the BY 448 hepatoma tending to be somewhat smaller.

Similar experiments were carried out with mitochondria prepared from " old " hepatoma transplants which contained some necrosis. It is evident from Table II that such mitochondrial preparations from the hepatomas BY 446 and BY 463 gave less satisfactory results than those from the " young " transplants of the same tumours illustrated in Table I. Glutamate oxidation was about half as effective and the P : O ratios were decreased. In the case of hepatoma BY 448 the present results with the " old " transplants were of the same unsatisfactory kind as those obtained with " young " transplants of this tumour ; however, no difference in glutamate oxidation between the mitochondria from " old " and " young " BY 448 transplants could be observed.

The conclusion to be drawn from the results presented in Tables I and II is that " young " transplants should be used for the study of the mitochondrial activities of rat hepatomas and that, if this condition is satisfied, some hepatomas yield mitochondria with an oxidative phosphorylation approximating that of normal liver mitochondria, while other hepatomas may yield particles which are deficient regardless of the fact whether " young " or " old " transplants are used.

TABLE III.—*ATPase Activity of Mitochondria from " Young " and " Old " Transplants of Rat Hepatoma BY 463*

Assay in the presence of Mg^{2+}

Condition transplant (generation)	Mitochondrial nitrogen/flask (mg.)	DNP (10^{-4} M)	μg. phosphorus released after 7·5 min.	μg. phosphorus released after 15 min.
" Young " (2)	0·05	—	9	17
" Young " (3)	0·16	—	20	32
		+	38	66
" Old " (5)*	0·10	—	68	110
		+	75	135

* Compare Table V : BY 463¶.

The effect of the age of the transplant (" young " *versus* " old ") is further illustrated in Table III for the Mg^{2+}- and DNP-activated ATPase of the mitochondria from the hepatoma BY 463. Although the ATPase activity of these particles (like that of the BY 225 mitochondria (Emmelot *et al.*, 1959)) when isolated from " young " transplants, is less completely latent than in the case of rat liver mitochondria, the enzymic activity is, nevertheless, less pronounced and the effect of DNP more significant than with mitochondria isolated from " old " transplants from the same hepatoma. These differences are of interest in view of the results reported below.

* Recently Borst and Slater (1960) reported that heart mitochondria oxidize glutamate as α-ketoglutarate after an initial transamination with oxaloacetate, as shown *inter alia* by the complete (97 per cent) inhibition of glutamate oxidation in the presence of malonate. Glutamate oxidation by isolated mitochondria therefore does not constitute proof of the presence of glutamic dehydrogenase, since oxidation may proceed in the absence of the latter enzyme by the above mechanism. Compare the absence of the enzyme in the Novikoff hepatoma and the comment by Emmelot *et al.* (1959). In the present hepatoma mitochondrial preparations glutamate oxidation was inhibited for only 50 per cent by malonate, indicating the involvement of the glutamic dehydrogenase in the oxidation.

Fatty acid oxidation

The ability of the mitochondria from " young " transplants of the hepatoma BY 225, BY 446 and BY 463 to oxidize fatty acids (pH 7·4) is illustrated in Fig. 1 for hexanoate. For fatty acid oxidation to occur the fatty acids have first to be converted to their corresponding acylcoenzyme A derivatives (Lynen and Ochoa, 1953). This activation reaction needs ATP, and if the latter is missing or present below some critical level as a result of the poor phosphorylative efficiency (high ATPase activity) of the mitochondria, fatty acid oxidation will be impossible. Hence the failure to oxidize fatty acids need not be due to the absence of the enzymes of the β-oxidation cycle but may result from a lack of ATP.

It was, therefore, according to expectation to find that the mitochondria from the " old " transplants of the BY 225, BY 446 and BY 463 hepatomas, and those from " young " and " old " transplants of the BY 448 hepatoma, all showing low P : O ratios, were unable to oxidize fatty acids, in contrast to the mitochondria from " young " transplants of the former three hepatomas. Table IV contains a summary of these results. Since fatty acids may, to a variable extent depending on their chain length (Pressman and Lardy, 1956 ; Avi-Dor, 1960), induce mitochondrial ATPase activity, a disorganization that can be counteracted by serum albumin, hexanoate has been added in a number of experiments in the form of its albumin complex. To this end hexanoate was dissolved in a 5 per cent serum albumin solution which had been thoroughly dialyzed before use in order to remove oxidizable substrates. An aliquot thereof, corresponding to 2 μmoles hexanoate and a final concentration of 0·3 per cent albumin was added to the respirometers. The presence of albumin, however, did not facilitate the hexanoate oxidation by

TABLE IV.—*Summary of Enzymic Properties of Mitochondria from Transplanted Rat Hepatomas*

In parentheses the experiments with " old " transplants.

Hepatoma	Transplant generation	P : O ratio (glutamate oxidation)	Hexanoate oxidation
BY 252*	45–60	2·2–2·5	++
	[45–60	..	0†]
BY 463	2	1·9	++
	3	2·5	++
	[5	1·5	0]
BY 446	[12	0	0]
	13	1·6	0
	14	1·5	0
	15	2·4	(albumin added) : +
BY 448	[12	0·8	0]
	13	0·4	(albumin added) : 0
	14	0·7	0
BY 484‡	2	1·9	(albumin added) : ++
	4	2·6	

* Data from Emmelot *et al.* (1959).

† " Old " transplants of hepatoma BY 225, in contrast to " young " ones, were unable to oxidize octanoate ; hexanoate oxidation by mitochondria from the " old " transplants of this hepatoma has not been studied.

‡ Recent experiment not included in the text solid hepatoma (primary and transplanted).

the mitochondria from " young " BY 448 transplants, but did so with the BY 446 mitochondria which were otherwise not (very) active in oxidizing the fatty acid.

Since for the measurement of the P : O ratios and the fatty acid oxidation the same pools of mitochondria were used, the data summarized in Table IV suggest that for hexanoate activation (and oxidation) to be catalysed by a particular hepatoma-mitochondrial preparation the phosphorylation, accompanying the oxidation of glutamate in the presence of glucose-hexokinase, must at least exceed an efficiency corresponding to a P : O ratio of 1·6.

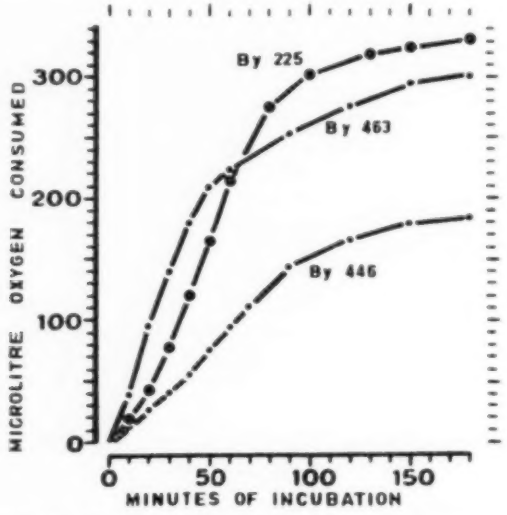


FIG. 1.—Hexanoate oxidation by mitochondria from the rat hepatomas BY 225, BY 463 and BY 446 (" young " transplants). Mitochondria from the same pools as used in the present experiments served also for the experiments illustrated in Table I. In one of the experiments (BY 446) serum albumin was present—see text. Incubation as described (Emmelot *et al.*, 1959); hexanoate (2 μmoles), L-malate (0·0003 M), ATP (0·0007 M) and DPN (0·001 M) present in addition. Oxygen consumption corrected for that obtained in the absence of hexanoate. 0·82, 1·25 and 1·02 mg. mitochondrial nitrogen were present in the experiments with BY 225 (transplant generation : *60*), BY 463 (*2*) and BY 446 (*15*) mitochondria, respectively.

The data listed in Fig. 1 allow for a calculation of the maximum rates of hexanoate oxidation observed in the present systems by taking into account the oxygen uptakes during those time intervals in which oxygen consumption proceeded fastest (*e.g.* for BY 463 mitochondria between 10 and 20 minutes). The microatoms oxygen consumed/mg. mitochondrial N/hour, thus calculated, amounted to 33·3, 23·2 and 10·7 for the mitochondria from hepatoma BY 225, BY 463 and BY 446, respectively. For the complete oxidation of two μmoles hexanoate to carbon dioxide and water (a trace amount of malate was always added) 347 μl. oxygen are required. This value is closely attained by the mitochondria from hepatomas BY 225 and BY 463 after a 3-hour period of incubation. The mitochondria from hepatoma BY 446 consumed only half of the theoretical

amount of oxygen during this period, at the end of which the oxygen uptake had come to a stop. In the latter case, however, albumin was present and this condition, though protecting mitochondrial integrity, might hamper the availability of hexanoate to the mitochondria by withholding it in the albumin complex. The slower rate of hexanoate oxidation by the BY 446 mitochondria in the presence of albumin (Fig. 1) as compared to that by the other hepatoma mitochondria in the absence of albumin thus, at least partly, might be accounted for. Support for the

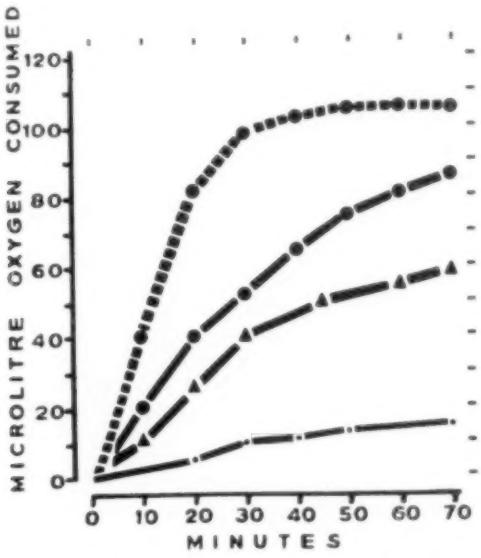


FIG. 2.—Palmitate oxidation by liver and hepatoma mitochondria. Incubation as indicated in Fig. 1, except that 0·4 μmole palmitate was now present. Dotted lines represent the oxygen uptake in the absence and solid lines in the presence of serum albumin (final concentration 0·3 per cent). Symbols:

●——● = mouse liver mitochondria (1·08 mg. N),
•——• = mitochondria from rat hepatoma BY 252 (0·83 mg. N) and from mouse hepatoma T 28612 (1·04 mg. N),
▲——▲ = mitochondria from two primary rat hepatomas (0·77 mg. N).

latter explanation came from experiments with normal mouse liver mitochondria incubated with palmitate in the absence and presence of albumin (Fig. 2). Since, however, in the latter case with palmitate and albumin present, the oxygen consumption reached the non-albumin level after 100 minutes of incubation (not illustrated in Fig. 2), whereas in the case of the BY 446 mitochondria (Fig. 1) hexanoate oxidation in the presence of albumin stopped long before the theoretical amount of oxygen was consumed, it might be concluded that the latter mitochondria suffered damage during their incubation which resulted in a loss of fatty acid oxidation either by a fall in the ATP level or by the inactivation of the fatty acid oxidizing enzymes.

Palmitate oxidation by the hepatoma mitochondria has only been studied in the case of the hepatoma BY 225. In the absence of albumin no oxidation whatsoever was observed, but in the presence of albumin a distinct, though exceedingly small, oxygen uptake could be recorded; a similar result was obtained with the mitochondria from the mouse hepatoma T 28012 (Fig. 2). As yet the only distinct evidence for palmitate oxidation by tumour mitochondria has been found in the case of two primary hepatomas induced by 4-dimethylaminoazobenzene in one rat (No. 391) of our inbred colony (Fig. 2). This result is mentioned here because it is, so far as we are aware, the first example to be presented for the ability of tumour mitochondria to oxidize palmitate.

Activation of the Mg^{2+}-dependent and inhibition of the DNP-activated ATPase of rat liver mitochondria by non-structurally-bound lipids isolated from rat hepatomas

In the introductory part of this paper it has been mentioned how we came to investigate the effect of the lipids, isolated from hepatoma homogenates, on the ATPase of normal rat liver mitochondria. After homogenisation of the tissues and application of the usual centrifugation for spinning down the nuclear fraction, the top-floating-lipid layer was collected from the tubes and washed several times by redispersion in fresh isotonic sucrose. Aliquots (0·1 ml.) of the final fatty dispersions were added to the incubation medium used for assaying the mitochondrial ATPase activity (Emmelot *et al.*, 1959). The concentration of the lipid was assessed by measuring the optical density at 520 mμ of 0·1 ml. of the final dispersion in 4·5 ml. sucrose. The latter values are listed in Table V together with the results of the ATPase assays carried out in the presence of Mg^{2+} and with DNP (10^{-4} M) absent or present. The effect of the lipids from the hepatomas BY 463, 446, 448 and normal rat liver on the ATPases of the normal rat liver mitochondria was tested at 27° C. and 37° C.

Lipid from rat liver (strain R-Amsterdam) showed little, if any, effect at 27° C. At 37° C. a slight increase in the Mg^{2+}-dependent and a small inhibition of the DNP-activated ATPase were observed (Table V). Since these effects were dependent on the presence of Mg^{2+} it followed that they were mediated by an effect on the mitochondrial structure. Neither at 27° nor at 37° C. could any effect be observed from the addition of the lipid prepared from the hepatoma BY 463 ("young" transplant). However, when the lipid from an "old" transplant of the same hepatoma was used, a marked increase in the Mg^{2+}-dependent ATPase of the rat liver mitochondria was observed at a low concentration of the lipid. It is of interest that the hepatoma mitochondria obtained from this same transplant showed a high free (Mg^{2+}-dependent) ATPase activity, as illustrated in Table III, whereas the hepatoma mitochondria from non-necrotic "young" transplants showed a much lower activity. Given its small concentration, a distinct effect with the lipid from hepatoma BY 446 (young transplants) was obtained, it consisted of a small increase in the Mg^{2+}- and a small inhibition of the DNP-ATPase activity at 27° C and greater effects at 37° C. The lipid from hepatoma BY 448, added in the lowest concentration of all, activated the Mg^{2+}- and inhibited the DNP-ATPase to a marked extent; the extra phosphate release normally brought about by DNP in the presence of Mg^{2+} was completely abolished at 37° C. and almost so at 27° C.

The effect of various concentrations of the lipid from hepatoma BY 225 is illustrated in Table VI. It is seen that, whereas at 27° C. only the highest concen-

TABLE V.—*Effect of Liver and Hepatoma Lipids on the ATPase Activity of Rat Liver Mitochondria in the Absence and Presence of DNP*

Lipids obtained from "young" transplants, except for the case with BY 463 marked ¶. DNP: 10^{-4} M. Assay in the presence of Mg^{2+}, unless otherwise stated, as described previously (Emmelot *et al.*, 1959): 0·1 ml. of the lipid dispersion added to 1·8 ml. of the reaction mixture. Mitochondria from 25 mg. of fresh weight of rat liver (strain R) added except in the first experiment in which the equivalent from 40 mg. of liver was present.

Source of lipid (concentration)*	Temperature of incubation (°C.)	μg. phosphor released: DNP absent after 7·5 min.	DNP absent after 15 min.	DNP present after 7·5 min.	DNP present after 15 min.
Control	37	15 (4)†	45 (21)†	56 (83)†	108 (130)†
Rat liver (0·090)	37	29 (4)†	58 (20)†	50 (82)†	75 (124)†
Control	27	3	6	47	83
BY 463 (0·155)‡	27	5	9	49	93
Control	27	3	7	50	80
BY 446 (0·020)	27	7	16	33	60
BY 448 (< 0·020)‡‡	27	12	27	23	37
Control	37	10	15	94	120
BY 463 (0·155)‡	37	10	15	87	120
BY 463 (0·020)¶	37	25	48	66	105
BY 448 (< 0·020)‡‡	37	44	62	48	65
BY 446 (0·020)	37	24	47	—	—

* Measured as indicated in the text.
† In the absence of Mg^{2+}.
‡ or ‡‡ Lipids from similar origin.
¶ Lipid from "old" transplant (generation: 5). The ATPase of the mitochondria from this transplant is illustrated in Table III.

TABLE VI.—*Effect of Various Concentrations of Lipid From Hepatoma BY 225 on the ATPase Activity of Rat Liver Mitochondria in the Absence and Presence of DNP*

0·1 ml. of the lipid dispersion in 4·5 ml. sucrose showed an optical density of 0·030 at 520 mμ.

BY 225 lipid (ml. added)	Temperature of incubation (°C.)	μg. phosphor released: DNP absent after 7·5 min.	DNP absent after 15 min.	DNP present after 7·5 min.	DNP present after 15 min.
Control	27	0	2	30	59
0·1	27	10	15	27	35
0·05	27	0	5	30	55
0·02	27	0	2	31	60
Control	37	4	7	62	124
0·1	37	26	53	39	65
0·05	37	18	50	60	100
0·02	37	7	22	60	110

tration of the lipid affected both the Mg^{2+}- and DNP-ATPase (activation and inhibition, respectively), all three concentrations of the lipid activated the Mg^{2+}-ATPase at 37° C., though with decreasing effect. At 37° C. the DNP-ATPase was markedly inhibited only by the lipid added in the highest concentration.

When the results obtained with the lipid from hepatoma BY 225 are compared with the data listed in Table V, it appears that the former lipid is about as active as that from hepatoma BY 446. It should be pointed out that in the present investigation no parallel experiments on the oxidative phosphorylation and fatty acid oxidation of the BY 225 mitochondria, such as has been the case with the other hepatomas, have been carried out. The results referred to above regarding the BY 225 hepatoma mitochondria have been obtained previously (Emmelot *et al.*, 1959), showing a certain degree of variation from one transplant to another.

TABLE VII.—*Histology and Some Enzymic Properties of Rat Hepatomas*

Rat hepatoma	Primary tumour*	Transplants*	Mitochondrial oxidative phosphorylation and fatty acid oxidation†	dCMP‡ deaminase activity
BY 225	Adeno—solid	Solid (fusiform cells)	"Normal"	High.
BY 446	Adeno	Solid	Somewhat less than "normal"	Moderate—high.
BY 448	Adeno—solid	Solid (few fusiform cells)	"Abnormal"	Moderate.
BY 463	Solid	Solid	"Normal"	High.
§BY 484	"	"	"	Moderate.

* Emmelot *et al.* (1961).

† "Normal" = activities under optimal *in vitro* conditions, approximating those of rat liver mitochondria (differences: ATPase, release of diphosphopyridine nucleotide: Emmelot *et al.*, 1959).

‡ dCMP = deoxycytidylic acid, Emmelot *et al.* (1961).

§ The dCMP deaminase activity of the 5th transplant generation of hepatoma BY 484 amounted to 37 and that of the 8th generation to 24–39 μmoles of deoxyuridylic acid formed per equivalent of soluble fraction derived from 1 g. of fresh tissue/60 min. (units). Calculated per mg.N of soluble fraction, the activity ranged from 2·9 to 3·9 μmoles deoxyuridylic acid formed per 60 min. Thymine-2-^{14}C catabolism was insignificant. Experimental conditions: Emmelot *et al.* (1961).

DISCUSSION

Attempt at classification of hepatomas.—From the data summarised in Table VII it follows that no relation between the histological and enzymic properties of the transplants of the hepatomas can be established.

Variability in hepatoma-mitochondrial behaviour.—The following observations may constitute a basis for an explanation of the variability encountered in mitochondrial behaviour among the rat hepatomas.

(i) When the activities of the Mg^{2+}- and DNP-ATPases of normal liver mitochondria, hepatoma mitochondria and liver mitochondria incubated in the presence of hepatoma lipid, are compared, the non-latency of the Mg^{2+}- and the deficiency of the DNP-ATPase of the two latter types of particles, in contrast to the corresponding properties of the normal liver mitochondria, stand out (Emmelot *et al.*, 1959;

Emmelot and Bos, 1957—in the figures of the latter paper P_i should be read as phosphor)

(ii) " Young " transplants of hepatoma BY 463 yielded mitochondria with low " free " (Mg^{2+}-) ATPase activity and a moderate DNP-activated ATPase, but " old ", necrotic, transplants yielded mitochondria with high " free " ATPase activity, DNP now causing hardly, if any, increment in ATP-splitting. The lipid obtained from the latter transplants activated the latent ATPase of normal liver mitochondria, in contrast to the lipid from the " young " transplants. Mitochondria from " old " transplants of BY 463 (and other) hepatomas, showed a poor oxidative phosphorylation and a lack of fatty acid oxidation.

(iii) The mitochondria from hepatoma BY 448 (" young " and " old " transplants) were deficient in fatty acid oxidation and in oxidative phosphorylation and exactly this hepatoma contained a lipid which was the most effective in activating the Mg^{2+}- and inhibiting the DNP-ATPase of normal liver mitochondria. The " young " transplants of hepatoma BY 463 contained a lipid devoid of any activity, whereas the mitochondria from this hepatoma showed a high oxidative phosphorylation and fatty acid oxidation. Finally, the BY 446 mitochondria were somewhat less efficient in the latter respects than the BY 463 mitochondria, and " young " transplants of the former hepatoma contained a lipid which was active in disturbing normal mitochondrial integrity, but less so than the lipid isolated from BY 448. Hence, a rather close parallel appeared to exist between the mitochondria-damaging effects of the lipids contained in the hepatomas and the activities of the mitochondria isolated from the corresponding hepatomas.

(iv) Complete removal of lipid droplets from the homogenates of hepatoma BY 225 has earlier been found (Emmelot *et al.*, 1959) to be required in order to obtain mitochondria with a relatively low " free " ATPase and a demonstrable DNP-ATPase.

(v) The hepatoma lipids were collected from the surface layer of the centrifuge tubes after centrifugation and thus represented non-structurally bound lipids (or lipoids, compare : Emmelot, Bos, and Reyers, 1960). By the very nature of this material,* the present results should be distinguished from those obtained by Pressman and Lardy (1956) and Avi-Dor (1960) with liver-microsomal fat or fatty acids and from those of Hülsmann, Elliott and Slater (1960) with unsaturated fatty acid released from liver mitochondria upon their ageing, though the mechanism of action might be the same in all cases. It might, however, be argued that our material, especially in the case of the " old " tumour transplants, contained breakdown products of mitochondrial and microsomal membranes.

In view of the above, we propose that adsorption of free lipids or related compounds from the hepatoma homogenates on to the mitochondria during the latter's isolation, may be responsible for the degree of lability inherent to the particles. This lability may range from very pronounced to slight, the present order being BY 448 $\gg$ BY 446 $>$ BY 463, as a result of the presence in the tumour cells of lipids possessing various degrees of mitochondria-damaging properties. The present hypothesis might also offer an explanation for the unsatisfactory behaviour of mitochondria isolated from " old " hepatoma transplants which contain much lipid material and possibly also for the variability encountered in the mitochondrial behaviour of other tumours (compare : Emmelot *et al.*, 1959).

* The present material designated as lipid has been found to contain fatty acids, cholesterol and some protein. The ATPase releasing activity is mainly residing in the long-chain fatty acid fraction.

Further support for the present conclusion may be adduced from previous experiments (Emmelot and Bos, 1957 ; Emmelot *et al.*, 1959—Table V, experiment marked*) which showed that in the presence of globulin the " free "—and DNP-ATPase of hepatoma and certain mouse liver mitochondrial preparations were decreased and enhanced, respectively, probably by binding of the lipid component. The sluggish or relatively poor effect of DNP on the hepatoma mitochondrial ATPase has led us earlier (Emmelot *et al.*, 1959) to postulate the possible existence of an endogenous inhibitor. The latter is now tentatively identified as to be of lipid nature, in view of the present results which demonstrated an inhibition of the DNP-activated ATPase by hepatoma lipid. The uncoupling agent found by Devlin and Pruss (1958) in the 25,000 $\times$ **g** supernatant of Novikoff hepatoma homogenates and apparently also in the mitochondria from this tumour, may also be of lipid nature.

Finally, we want to point out that in certain tumour homogenates the mitochondria may become contaminated by microsomal fragments—the phospholipide components (vesicles), containing high ATP- and DPN-splitting properties, which represent the double membranes of the endoplasmic reticulum *in situ*—especially in those cases in which high DPNase activities have been found inherent to certain tumour mitochondrial preparations (Emmelot, 1957 ; not in the case of BY 225 mitochondria : Emmelot *et al.*, 1959). Moreover, the possibility should be reckoned with that some tumour mitochondria *per se* possess a small intrinsic stability, a view that is supported by electron microscopical observations *in situ* (Emmelot *et al.*, 1959). Such particles might be especially vulnerable to the damaging effect of the lipids *in vitro* (if not already damaged by lipid *in situ*).

SUMMARY

Mitochondria were isolated from " young " and " old " transplants of three rat hepatomas, BY 463, BY 446 and BY 448, and studied in respect of oxidative phosphorylation (substrate : glutamate) and fatty acid oxidation (substrate : hexanoate). The results were compared with those obtained previously with mitochondria from the rat hepatoma BY 225. With all four hepatomas the oxidative phosphorylation and the fatty acid oxidation was decreased or lacking when the mitochondria had been isolated from " old " transplants. Mitochondria from the " young " transplants, however, gave favourable results in the case of the hepatomas BY 225 and BY 463, and somewhat less so in the case of BY 446. By contrast, the mitochondria obtained from " young " transplants of the hepatoma BY 448 yielded P : O ratios always less than unity and were unable to oxidize hexanoate even if serum albumin was added. The latter two deficiencies are probably related in that fatty acid activation was made impossible by a lack of a sufficient amount of ATP. The BY 448 hepatoma contained a lipid which, in a low concentration, activated the Mg^{2+}-dependent and inhibited the dinitrophenol-activated ATPase of normal rat liver mitochondria to a marked extent. A smaller effect was displayed by the lipid from hepatoma BY 446 but none at all by that from hepatoma BY 463. However, lipid isolated from " old " transplants of the latter tumour activated the latent ATPase of liver mitochondria and hepatoma mitochondria isolated from the latter transplants possessed high " free " ATPase activities, in contrast to the low to moderate free ATPase activities of BY 463 hepatoma mitochondria from " young " transplants. An adsorption of the active

lipids from the particular hepatoma homogenates onto the mitochondria during the latter's isolation, followed by structural and functional disarrangement, might account for the lability noted among the corresponding mitochondrial preparations. The latter problem is briefly discussed.

REFERENCES

Avi-Dor, Y.—(1960) *Biochim. biophys. Acta*, **39**, 53.
Borst, P. and Slater, E. C.—(1960) *Ibid.*, **41**, 170.
Devlin, T. M. and Pruss, M. P.—(1958) *Fed. Proc.*, **17**, 211.
Emmelot, P.—(1957) *Exp. Cell. Res.*, **13**, 601.
Idem and Benedetti, E. L.,—(1960) *J. biophys. biochem. Cyt.*, **7**, 393.
Idem and Bos, C. J.—(1957) *Brit. J. Cancer*, **11**, 148.
Idem, Bos, C. J., Brombacher, P. J. and Hampe, J. F.—(1959) *Ibid.*, **13**, 348.
Idem, Bos, C. J., Brombacher, P. J. and Reyers, I. H. M.—(1960) *Nature, Lond.*, **186**, 556.
Idem, Bos, C. J. and Reyers, I. H. M.—(1960) *Z. Krebsforsch.*, **64**, 52.
Idem, Hampe, J. F., Bos, C. J. and Reyers, I. H. M.—(1961) *Brit. J. Cancer*, **15**, 138.
Hülsmann, W. C., Elliott, W. B. and Slater, E. C.—(1960) *Biochem. biophys. Acta*, **39**, 267.
Lynen, F. and Ochoa, S.—(1953) *Ibid.*, **12**, 299.
Pitot, H. C. and Potter, V. R.—(1960) *Ibid.* **40**, 537.
Pressman, B. C. and Lardy, H.—(1956) *Biochim. biophys. Acta.* **21**, 458.

ON THE FAILURE OF TUMOUR HOMOGENATE INJECTIONS TO SIMULATE QUANTITATIVELY THE CHANGES IN CATALASE ACTIVITY IN THE TUMOUR-BEARING HOST

D. H. ADAMS

From the Cancer Research Department, London Hospital Medical College, London, E.1

Received for publication April 5, 1961

In recent years much work has been done on the catalase depressing action of fractions derived from tumour tissues. However the effects on liver catalase produced by these fractions, or by the injection of whole homogenates of tumour tissues, have seldom been compared quantitatively with those observed in the tumour-bearing animal. In fact there now seems considerable doubt whether injections of tumour homogenates or fractions are capable of quantitatively simulating the action of the tumour on its host. In the tumour-bearing mouse most investigators seem to agree that catalase activity falls progressively during tumour growth, and remains low until the death of the animal. Recently, however, Kampschmidt, M. E. Adams and McCoy (1959) found that when daily injections of tumour fractions were given to mice, their catalase activity fell sharply, but after a few days rose again while the injections were still being given. Adams (1951) injected mice daily with homogenates of Sarcoma 37 for 4 days without observing any restoration of the lowered catalase level. It may be, however, that the injections were not carried on for a sufficiently long period. Adams and Roe (1953) found that repeated daily injections of methyl butter yellow produced a depression in catalase activity, followed by a return of the activity to normal by the 7th day.

It also seems that injected tumour homogenates and fractions do not depress catalase activity to the low levels found with tumour bearing animals. However an accurate assessment of the situation is made difficult by the use of excessively large tumours by some investigators, and also by the fact (Adams and Burgess, 1957) that the catalase activity measured may be principally that of the extra-particulate cytoplasm (EPC), or that of the EPC + granules, depending on the technique employed for the reparation of the liver tissue. In this laboratory the following observations relating to mice bearing Sarcoma 37 have been made. There is a rapid decrease in catalase activity in EPC and granule fractions until the tumours have reached 5–10 per cent of body weight. Subsequently the decrease continues but at a slower rate. In hosts bearing tumours of about 10 per cent of body weight the total (EPC + granule) catalase activity is about 50 per cent of normal. The EPC catalase activity is about 20 per cent of normal, and consequently the granule/EPC catalase distribution ratio increases to about 8 in females and 6·5 in males. (Normal values with the homogenising medium used are about 5 in females and 3–3·5 in males). It is of interest in this connection that Thomson and Klipfel (1958) found that although total catalase activity fell markedly in tumour-

bearing animals, the distribution of liver catalase within the intracellular granules did not alter. However, these authors did not study the distribution of the enzyme between granules and EPC.

Single injections of Sarcoma 37 homogenate were found to decrease the catalase level in both granule and EPC fractions, and to increase the granule/EPC distribution ratio, but in both males and females these changes were not so pronounced as in tumour-bearing animals (Adams, 1959).

The purpose of the present paper is to show whether a closer simulation of the tumour-bearing animal can be obtained with multiple injections of tumour homogenates.

MATERIALS AND METHODS

Animals.—Young adult mice of the CBA strain were used. The animals were bred in this laboratory by brother-sister mating. They were fed on rat cubes obtained commercially.

Preparation of liver fractions and estimations of liver catalase activity.—Catalase estimations were made on whole liver homogenates, and on granule and EPC fractions. The method of estimation of catalase activity has been fully described in previous publications (Adams, 1950, 1952) and also the preparation of the homogenates and fractions (Adams and Burgess, 1957, 1959*a*). Ethanol (final concentration 0·01 M) was added to all catalase-containing solutions, to prevent loss of catalase due to "Complex II" formation (Chance, 1950; Adams and Burgess, 1959*b*).

Tumour.—Sarcoma 37 was obtained originally from the Imperial Cancer Research Fund Laboratories and maintained by serial passage in this laboratory. Tumour (and normal) tissues were coarsely homogenised in normal saline solution and injected subcutaneously. The doses cited are weights of tissue (as homogenate) contained in the inoculum.

RESULTS

It was decided at first to study the effects of daily injections of a chemical agent on liver catalase activity and intracellular distribution of catalase. Since (Adams, 1960) animals injected with thiourea had been found to have low catalase activities, a comparison was made of the effects of single and multiple doses of this substance on the catalase activity and intracellular distribution in male mice (Fig. 1). The results show that single doses of 6 μmoles or 10 μmoles of thiourea produced a depression of catalase activity in both granule and extra-particulate cytoplasmic (EPC) fractions without change in the granule/EPC catalase distribution ratio. By the 4th or 5th day the catalase activity had returned to normal. It is evident from Fig. 1 that continuous daily injections of 6 μmoles of thiourea gave results resembling those seen after the injection of the single dose of 10 μmoles. Fig. 2 shows the effect of the continuous daily injection of varying doses of mixed normal liver-spleen-kidney homogenate prepared from 101 strain mice on the catalase activity and distribution ratio of CBA female mice. A daily dose of 25 mg. produced a fall in catalase activity followed by a return to normal by the 4th day. Fifty mg./day produced a greater depression, and the activity began to rise again after three days. Some evidence of a rise in catalase activity from the lowest point was

also obtained with a dose of 100 mg./day but since the mice did not tolerate this dose well, the experiment was not continued beyond the 5th day. At no time was there any significant change in the granule/EPC catalase distribution ratio.

A similar experiment was done using a homogenate of Sarcoma 37 tissue. Daily injections of 25 mg. produced little effect on the granule catalase activity, but significantly reduced the EPC level. Fifty mg./day produced depressions of activity in both fractions followed by restoration of the granule catalase activity

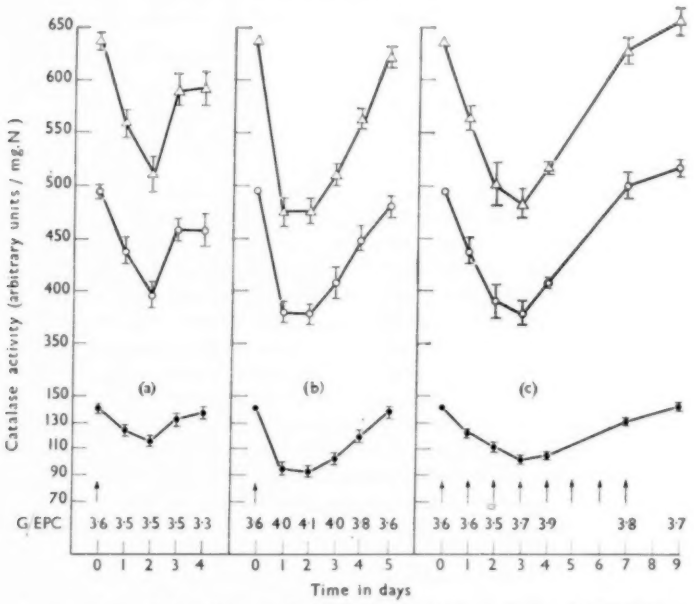


FIG. 1.—A comparison of the effects on liver catalase activity and granule/EPC distribution ratio of single and continuous daily injections of thiourea. Doses given at times indicated by ↑. Graph (*a*) 6 μmoles (*b*) 10 μmoles (*c*) 6 μmoles/day. Total catalase (△- - -△), granule catalase (○- - -○) and EPC catalase (●- - -●) activities are given in this and subsequent figures as arithmetic means ± Std. error of mans. Eight animals/group.

to normal by the 5th day, but the EPC catalase remained depressed. The results of the 100 mg./day dose showed a rise from the minimum level by the fifth day. As with the normal tissue, this dose was not well tolerated by the mice. Unlike the normal tissue experiments, the granule/EPC catalase distribution ratio was increased to about 6·0–6·5, and this occurred independently of dose.

In a further experiment male mice were injected daily with 50 mg. doses of homogenised S37, and another group with the same dose of liver-spleen-kidney homogenate. As with the female mice the normal tissue produced a depression in catalase activity without change in the granule/EPC distribution ratio. The rise of catalase activity from the lowest point was more pronounced than in the females receiving the same dosage. An increase in catalase granule/EPC distribution ratio was found in the males treated with S37, and there was a sharp rise in catalase

activity from the lowest point. It appeared in this experiment that the distribution ratio also was returning towards normal on the 3rd and 4th days.

DISCUSSION

The results have shown clearly that while multiple injections of Sarcoma 37 produced a depression in liver catalase level and an increase in catalase granule/EPC distribution ratio the tumour injections failed in a number of respects to

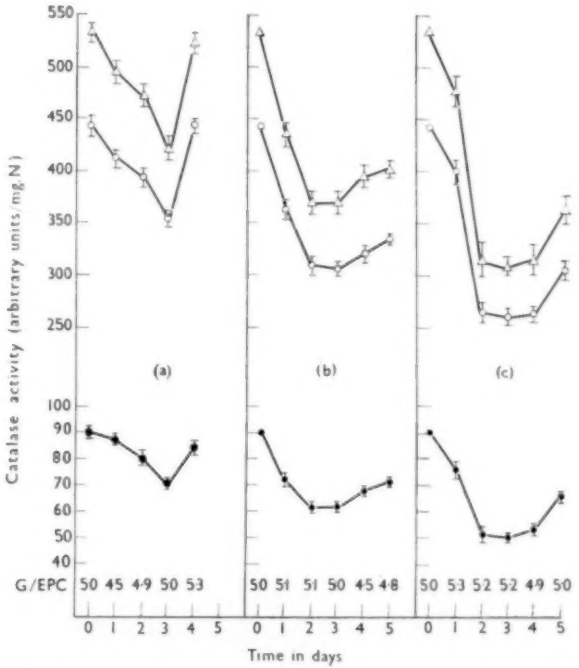


FIG. 2.—The effect of the continuous daily injection of normal tissue (mixed LSK) homogenate on the catalase activity and granule/EPC distribution ratio in CBA female mice. (*a*) 25 mg./day (*b*) 50 mg./day (*c*) 100 mg./day.

simulate the picture in the tumour-bearing mice. Even when the dose was raised to the maximum tolerated by the animals, Sarcoma 37 homogenate failed to increase the catalase granule/EPC distribution ratio beyond 6·0–6·5 in females and 4·9 in males compared with about 8·0 and 6·5 in tumour-bearing females and males. Also the total catalase activity was still 60 per cent of normal as against about 40 per cent or less in tumour-bearing animals (Adams, 1959). The increase in distribution ratio was independent of dose over the range studied.

The experiments with thiourea showed that the catalase activity of treated animals was able to return to normal while the injections were still being given.

This suggests that the rate of catalase synthesis can be increased considerably to meet extra demands on the enzyme. There must however be limits to the ability of the system to restore catalase activity to normal, during the continuous injection of agents which reduce the activity. Thus, although in the tissue homogenate injection experiments there was evidence that catalase activity rose from the lowest point during the injection of doses up to 50 mg./day, it was not clear whether

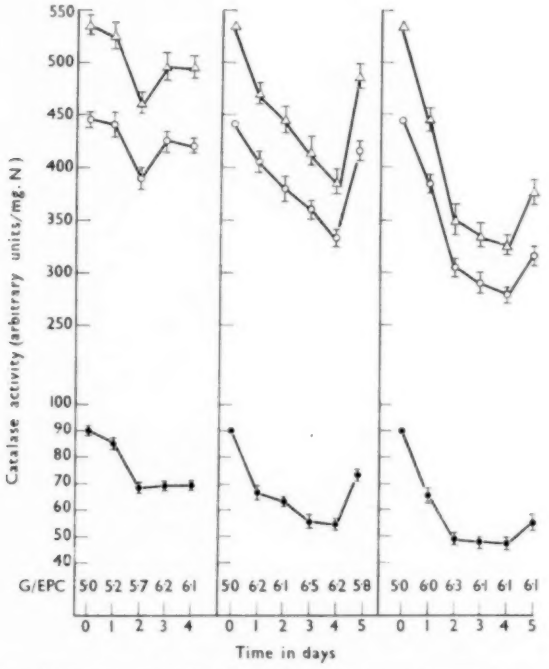


FIG. 3.—The effect of continuous daily injections of S37 tissue homogenate on the catalase activity and granule/EPC distribution ratio in CBA female mice. (*a*) 25 mg./day (*b*) 50 mg./day (*c*) 100 mg./day.

this occurred during the injection of 100 mg./day. There was, it is true some evidence of a rise from the lowest point, but it was not possible to continue the experiments beyond about 5 days. In any event, however, if the catalase depression in tumour-bearing animals is due to release of a specific tumour agent plus tumour breakdown products it follows that the liberation of at least 100 mg./day of tumour substance would be required to prevent a return of catalase activity to normal. But it seems that not even this dose will alter catalase activity and distribution ratio to the values reached in tumour-bearing animals. If catalase levels following the continuous injection of tumour tissue are compared with those found in tumour-bearing animals (Adams, 1959), 100 mg./day of S37 is equivalent to the presence in the host of a tumour weighing one gram or less. Apart from the fact

that 100 mg. of tissue/day was the maximum dose tolerated by the animals, it seems unreasonable to suppose that tumours are capable of releasing this amount of breakdown products per day, especially in the early stages of growth. Further the loss in total catalase activity following the injection of S37 homogenate is if anything less than that following the injection of normal LSK homogenate, leaving little or no room for the contribution of a specific tumour agent. It may be

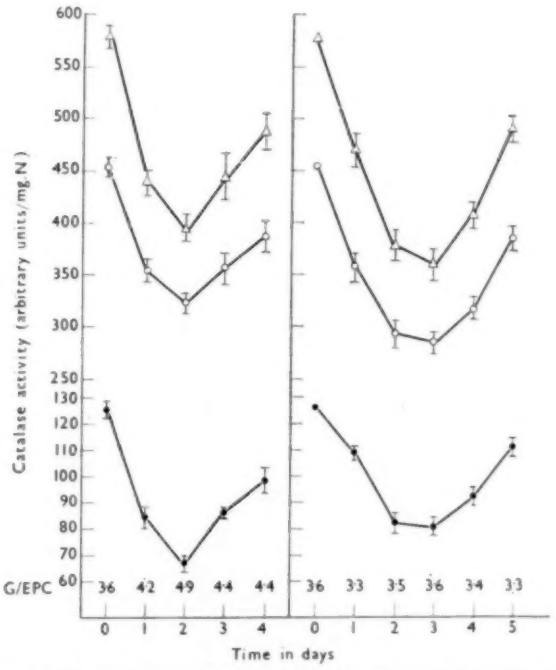


FIG. 4.—The effects of the continuous daily injection of (*a*) 50 mg. S37 homogenate, (*b*) 50 mg. normal LSK as homogenate, on the catalase activity and granule/EPC distribution ratio of CBA male mice.

argued that the catalase depressing effect of the tumour is mainly due to the release of a specific agent, but as already stated, Kampschmidt, M. E. Adams and Mc'Coy (1959) found that catalase activity rose to normal while tumour fractions were being injected, so that either this agent must be absent from, or present in insufficient amount in such fractions, or some other effect of the growing tumour must be sought for.

One point of interest arising from this paper is that the EPC catalase activity of the CBA males was not decreased to a greater extent than that of females by the injection of tumour homogenate. This contrasts with earlier results reported using an albino strain (Adams, 1950, 1951). Recently (Adams, 1960, and unpublished experiments) evidence has been obtained that different androgenic hormones are present in the CBA and albino strain of mice. CBA female mice injected

with testosterone had EPC catalase levels elevated about halfway to the male level. The EPC catalase level of females of the albino strain under the same conditions was elevated to the male level. When 17 methyltestosterone was injected into females of both strains, the EPC catalase activity of the CBAs rose to the male level, and of the albinos to considerably above the male level. In view of the evidence (Adams, 1951) that a tumour agent interferes with the action of hormones on catalase, one explanation of the greater resistance of the catalase in CBA mice may be that they secrete a different androgenic hormone.

It was suggested (Adams, 1959) that the effects on catalase produced by tumour homogenate injections could be divided into two parts : a depression in total catalase activity, which appeared quite non-specific, and a change in catalase distribution. The present work lends additional support to this view, and it may well be asked what relevance the ability of tumour homogenates or fractions to *depress* catalase activity has to the lowered catalase activity in the tumour-bearing animal. It is therefore proposed to continue this enquiry on the supposition that an agent present in tumours induces changes in the host which, in conjunction with certain nutritive demands of the tumour, lead to the observed changes in host catalase activity and distribution.

SUMMARY

(1) The changes in mouse liver catalase activity and distribution following multiple injections of Sarcoma 37 homogenate resemble, but do not quantitatively match, those seen in S37-bearing mice.

(2) It is concluded that a tumour agent alone can hardly be responsible for the observed changes in tumour-bearing animals.

My thanks are due to Dr. M. H. Salaman for his interest and to Miss Iris Harris for skilled technical assistance. The expenses of this research were partly defrayed out of a block grant from the British Empire Cancer Campaign.

REFERENCES

Adams, D. H.—(1950) *Brit. J. Cancer*, **4**, 183.—(1951) *Ibid.*, **5**, 115.—(1952) *Biochem. J.*, **50**, 486.—(1959) *Brit. J. Cancer*, **13**, 704.—(1960) *Biochem. J.*, **74**, 141.

Idem and Burgess, E. A.—(1957) *Brit. J. Cancer*, **11**, 310.—(1959*a*) *Biochem. J.*, **71**, 340.—(1959*b*) *Enzymologia*, **20**, 341.

Idem and Roe, F. J. C.—(1953) *Brit. J. Cancer*, **7**, 509.

Chance, B.—(1950) *Biochem. J.*, **46**, 387.

Kampschmidt, R. F., Adams, M. E. and McCoy, T. A.—(1959) *Cancer Res.*, **19**, 236.

Thomson, J. F. and Klipfel, F. J.—(1958) *Ibid.*, **18**, 229.

SURVEY OF PAPERS

MILLAR has investigated possible factors to account for the high incidence of gastro-intestinal cancer in a part of central Wales. He gives reasons for suspecting that in this region polonium-210 may gain access to the human body in beef and mutton from local hill cattle and sheep which have grazed widely upon upland pastures (p. 175).

CORNES, JONES and FISHER conclude that there is no significant association between the development of carcinoma and leukaemia, multiple myeloma and malignant lymphoma, having found only 16 carcinomas in 585 patients dying from those diseases. They compare the low incidence of carcinoma in this series with a higher incidence of carcinoma found in patients with primary malignant lymphomas of the small or large intestine (p. 200).

KREYBERG has tested for two years the criteria for the typing of lung tumours tentatively proposed by the group of experts convoked by the World Health Organisation in Oslo in 1958. He reports on the usefulness of the criteria and discusses some difficulties which may be encountered (p. 206).

KREYBERG and SAXÉN find support for the thesis that the main increase in lung cancer in large areas of the world is caused by an increase in the epidermoid and small cell analplastic types from a comparison of large series of cases from Finland and Norway (p. 211).

CARROLL reports studies of the bronchial epithelium present in 92 resected primary lung tumours in an attempt to clarify the relationship of squamous metaplasia, basal cell hyperplasia and intraepithelial carcinoma of the bronchial epithelium to invasive primary lung cancer (p. 215).

SALM reports a case of vaginal botryoid sarcoma in a girl of 2 years who died 7 months after the onset of the disease (p. 220).

COHEN and HOE give a detailed account of 170 cases of primary cancer of the oesophagus seen in Singapore between 1948 and 1957 (p. 226).

FRASER, SPICER, WILLIAMS and YOUNG have studied mammotrophic activity in unextracted urine and serum from normal women. The activity is defined as the capacity to stimulate proliferation of the mammary glands in hypophysectomised weanling male mice of a particular strain when these are injected at the same time with oestrone and progesterone. Such activity was rarely detectable in the urine but was present in most samples of serum. They found very great variation in responsiveness of the mice which they were unable to reduce by modifying the experimental procedures though they did manage to simplify the test considerably. They conclude that the method is capable of detecting mammotrophic activity but is not suitable for its accurate measurement (p. 243).

BOYLAND, CHARLES and GOWING have studied the induction of carcinoma of the cervix in mice by the application of various spermicides and known carcinogens in a polyethylene glycol base. They found so many tumours in the control series treated with the Carbowax alone that the possible carcinogenic activity of the spermicidal compounds could not be assessed (p. 252).

BIELSCHOWSKY and BIELSCHOWSKY have compared the susceptibility of the skin of pituitary dwarf mice to the action of DMBA with that of their phenotypically normal litter mates. They find that tumours develop in all controls but in only 60 per cent of the dwarfs even when both groups are treated with equal amounts. The latent period is always longer in dwarfs (p. 257).

HOWELL finds that 9,10-dimethyl-1,2-benzanthracene in oil is effective in producing breast carcinoma in female rats when administered by intranasal instillation. He also describes the changes in the lungs, including a small number of tumours, resulting from the same treatment (p. 263).

BIANCIFIORI, BONSER and CASCHERA have given virgin milk-agent-free C3H mice a limited dose of four chemical carcinogens by mouth. They found that granulosa cell ovarian tumours were induced in high yield by DMBA and MC; luteomas were induced by BP and DBA, and mammary tumours were induced by all four chemicals in descending order DMBA, MC, DBA and BP.

UMI

Control virgins kept 5 in a cage underwent 14 per cent of psuedopregnancies and occasionally developed small luteomas and granulosa cell tumours of the ovary spontaneously.

It is suggested that the granulosa cell tumours arose in areas of thecal proliferation and the luteomas in damaged follicles and that the occurrence of mammary tumours is related to the fact that virgin mice undergo pseudopregnancy (p. 270).

PERRY finds that adrenalectomy protects the liver of male Wistar rats against the carcinogenic effect of 2-acetylaminofluorene as long as the accessory adrenal tissue does not exceed a critical size (p. 284).

ARCOS and GRIFFITH find that potentiation of the hepatocarcinogenic effect to rats of 3′-methyl-4-dimethylaminoazobenzene by its relatively inactive 2-methyl isomer occurs when the two compounds are fed for 7 months in 1 : 2 ratio. There is no synergism, however, when the ratio is 1 : 1 following doubling of the level of the former dye. The 2-methyl derivative is neither synergistic nor antagonistic to 4–dimethylaminoazobenzene when fed previously for 3 months alone (p. 291).

LAWS and YATES compare the behaviour in tissue culture of explants of regenerating rat liver after partial hepatectomy from animals fed with the carcinogen 2-acetylaminofluorene and normal controls (p. 299).

NAKAMURA and METCALF have made a quantitative cytological study on thymic lymphoid cells in normal, preleukaemic and leukaemic AKR mice, comparing the findings with those in C3H mice (p. 306).

METCALF and NAKAMURA compare the results of cytological analysis of thymuses of AKR mice and testing for the presence of leukaemic cells by intramuscular transplantation into AKR recipients in an investigation into the nature of the increased percentage of primitive cells in the thymus immediately before the appearance of spontaneous thymic lymphomata (p. 316).

TOTH, DELLA PORTA and SHUBIK report the rapid occurrence of malignant lymphomas in Swiss mice given urethan in the drinking water (p. 322).

CSABA, ÁCS, HORVÁTH and MOLD have studied the origin and function of mast cells in the vicinity of induced, homologous and heterologous tumours in mice (p. 327).

HARTVEIT has investigated the factors concerned in the survival time of mice after intraperitoneal injection with Ehrlich's ascites carcinoma (p. 336).

WILLIAMS and JORANGER have developed sublines of two mouse ascites tumours, the Ehrlich carcinoma and Sarcoma 180, exhibiting resistance to triethylene melamine by consecutive passage of cells in mice treated with the drug (p. 342).

DRAYTON finds that the loss of biological activity which ensues after partially purified Rouse I virus preparations are treated with lipid solvents is correlated with the liberation from the virus particles of material which absorbs strongly in the ultraviolet between 2700 Å and 2800 Å, and is very likely protein. The integrity of at least some of the protein bound to lipid in the extra-nucleoid virus material would appear to be essential for infectivity (p. 348).

POWELL describes the effects of dextran-saline on HeLa cells. After recovery from the immediate effects of treatment, the cells proliferate for some days but then degenerate (p. 354).

CALCUTT and COATES have examined the effects of some polycyclic hydrocarbons and croton oil on mouse skin — SH levels. Non-carcinogenic hydrocarbons have little effect, whilst carcinogenic hydrocarbons and croton oil cause an elevation of mouse skin — SH levels (p. 360).

QUAGLIARIELLO, TANCREDI, FEDELE and SACCONE find that the urine from patients with bladder cancer usually contains 3-hydroxyanthranilic acid, kynurenine and anthranilic acid. The excretion of 3-hydroxyanthranilic acid is reduced by treatment with large doses of nicotinamide. They think it evident that there is a relationship between metabolites of tryptophan and cancer of the bladder (p. 367).

EMMELOT and BOS have studied isolated mitochondria from transplanted rat hepatomas in respect to oxidative phosphorylation and hexanoate oxidation (p. 373).

ADAMS concludes that a tumour agent alone can hardly be responsible for the observed changes in liver catalase activity and distribution found in S37-bearing mice (p. 386).

The Marconi 250kV Deep Therapy Set

A double installation in a British hospital where two sets are controlled by a single operator.

Designed and Constructed to give Constant Potential and Constant Operation

Constant Potential provides a high röntgen output with a high half value layer, thus reducing the skin dose and increasing the depth dose during both moving and static beam therapy.

A MARCONI set provides many years of continuous use. Many of these sets are installed both in Britain and overseas with an aggregate of more than a hundred years of trouble-free service.

MARCONI INSTRUMENTS

SPECIALISTS IN THERAPEUTIC AND DIAGNOSTIC X-RAYS

MARCONI INSTRUMENTS LTD., ST. ALBANS, HERTFORDSHIRE. TELEPHONE: ST. ALBANS 59292

London and the South: English Electric House, Strand London, W.C.2. Tel: COVent Garden 1234
Midlands: 24, The Parade, Leamington Spa. Tel: 1408 *North:* 23–25 Station Square, Harrogate. Tel: 67455

WORLD-WIDE REPRESENTATION XR33

UMI

SAFER THAN MORPHINE

for long term analgesia

In chronic, severely painful states such as terminal carcinoma, Narphen controls pain as efficiently as morphine, but without causing somnolence or unconsciousness.

Patients can therefore enjoy freedom from pain while mentally alert and without the prospect of a prolonged vegetative existence.

Because tolerance develops far more slowly than with morphine, Narphen is safer for prolonged treatment.

NARPHEN

for deep analgesia without narcosis

Narphen is SNP brand Phenazocine(2′-hydroxy-5,9-dimethyl-2-phenethyl-6,7-benzomorphan hydrobromide). Available in 1·1 ml. ampoules each containing 2 mg. phenazocine hydrobromide per ml. in aqueous solution. Boxes of 10 & 100 ampoules. Basic N.H.S. cost 15/- and 120/-. Exempt from Purchase Tax.

Subject to Dangerous Drugs Regulations

SNP **Smith & Nephew *Pharmaceuticals* Limited**
Welwyn Garden City · Hertfordshire

UMI

The SERVICE

built for a lifetime of constant use

The 'Service' is without doubt the finest student laboratory microscope today. The simplicity of its design is allied with the highest standards of construction, resulting in a high quality instrument easy to work with and capable of giving a lifetime of satisfactory service in constant use. The comprehensive range of accessories, designed to make the 'Service' suitable for more complex work is of equally high quality. See the Watson 'Service' before you decide on your next microscope.

WATSON

W WATSON & SONS LIMITED · BARNET · HERTFORDSHIRE

UMI

Biochemistry and Biological Actions of Steroids

EDITED BY

K. FOTHERBY *et al.*

The tenth *Memoir* of the Society for Endocrinology includes the papers given at the symposium on the relation of the endocrine glands to cancer, held at a joint meeting of the Society and the Acta Endocrinologia Congresses. *45s. net*

CAMBRIDGE
UNIVERSITY PRESS

Smoking and Lung Cancer

TWO LEAFLETS

Smoking—*A Medical Officer of Health speaks* (*S 111*)

J. L. Burn, MD, *Medical Officer of Health, Salford, Lancs.*

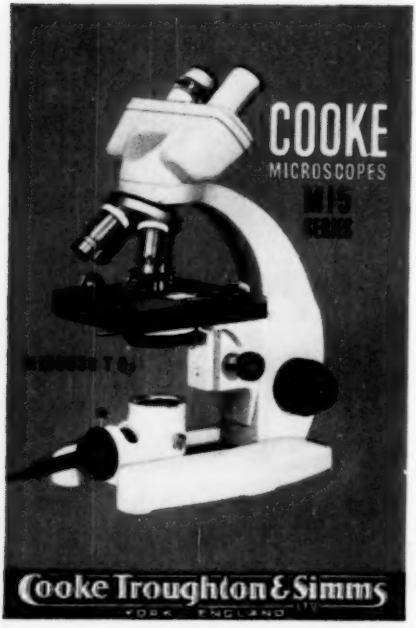
2d. a copy **15s.** a hundred

Cigarette Smoking—
What harm does it do? (*S 116*)

6d. a copy **30s.** a hundred

Send for free specimen copies of these and other leaflets and posters on smoking and lung cancer

The Chest and Heart Association
Tavistock House North
Tavistock Square, London WC1



British Empire Cancer Campaign

A SURVEY OF CANCER IN LONDON

REPORT OF THE CLINICAL CANCER RESEARCH COMMITTEE

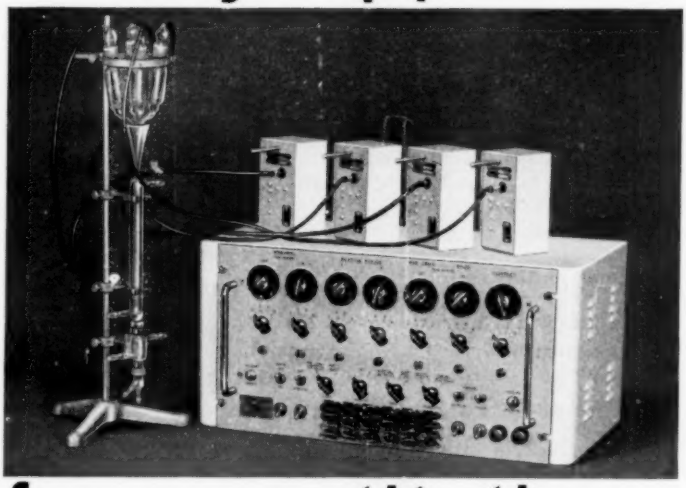
by W. L. HARNETT,
C.I.E., M.D., F.R.C.S,
Medical Secretary to the Committee.
With a FOREWORD by the
RT. HON. LORD HORDER,
G.C.V.O., M.D., F.R.C.P.,
and an INTRODUCTION by
SIR HENEAGE OGILVIE,
K.B.E., D.M., M.Ch., F.R.C.S.

Pp. vi + 834 with 22 figures in the text.

Price bound in paper covers 45/–, cloth 50/–.
Packing and postage.
Inland 3/9d, Canada 5/9, U.S.A. 7/6d.

BRITISH EMPIRE CANCER CAMPAIGN
11 Grosvenor Crescent, London, S.W.1

Automatic Biological Assay Apparatus



for quantitative measurement of the activity of drugs

This new apparatus greatly improves the accuracy, speed and convenience of measurements made on isolated organs and by being fully automatic releases skilled technicians for other work. Typical examples of its use are:

Standard assay of posterior extract on guinea pig uterus.
Test activity of an antispasmodic drug on rabbit intestine.
Four-point assay of histatamine on guinea pig ileum.

Send for Leaflet No. 801/E4

CASELLA (ELECTRONICS) Ltd., Haxby Rd., York. Tel. 24112

CASELLA ELECTRONICS

(Now controlled by Cooke, Troughton & Simms Ltd., Haxby Rd., York) Members of the Vickers Group

31

11-8-61

Treatment of Cancer and Allied Diseases

Edited by GEORGE T. PACK, M.D., F.A.C.S., *Associate Professor of Clinical Surgery, Cornell University; and* IRVING M. ARIEL, M.D., F.A.C.S., *Associate Clinical Professor of Surgery, New York Medical College.*

This important series — four volumes of which are now published — will provide a complete working library in nine volumes on the clinical aspects of tumour therapy. Nowhere else in the literature can there be found such a detailed and complete repository of every therapeutic method available to-day. **Volumes in the series are sold separately.**

VOLUME 4—TUMOURS OF THE BREAST, CHEST AND ESOPHAGUS

In this volume sixty-seven authorities deal with every type of neoplasm of the breast, chest and esophagus. The book is a comprehensive textbook of surgical technique, covering many operations and the management of the surgical patient. It gives detailed coverage also of modern radiation therapy and hormonal and chemotherapy. Hundreds of illustrations, some in colour, greatly enhance the text.

667 pages **650 illustrations** **£12 net**

PITMAN MEDICAL PUBLISHING CO. LTD.

39 PARKER STREET · LONDON · W.C.2

CONTENTS—*contd.*

POWELL, A. K.—The Effects of Dextran-saline ("Dextraven") upon Cells Cultivated *in vitro*. The Response of Actively Growing HeLa Carcinoma Cells 354

CALCUTT, G. AND COATES, JOAN.—The Sulphydryl Levels of Mouse Skin During Treatment with Polycyclic Hydrocarbons and Croton Oil 360

QUAGLIARIELLO, E., TANCREDI, F., FEDELE, L. AND SACCONE, C.—Tryptophan-nicotinic Acid Metabolism in Patients with Tumours of the Bladder. Changes in the Excretory Products after Treatment with Nicotinamide and Vitamin B_6 367

EMMELOT, P. AND BOS, C. J.—Studies on Isolated Tumour Mitochondria: Oxidative Phosphorylation and Fatty Acid Oxidation by Rat Hepatoma Mitochondria 373

ADAMS, D. H.—On the Failure of Tumour Homogenate Injections to Simulate Quantitatively the Changes in Catalase Activity in the Tumour-bearing host 386

SURVEY OF PAPERS i

RATES FOR

ADVERTISEMENTS

IN THIS JOURNAL

Whole page	...	**£10 0 0**
Half page	...	**£5 10 0**
Quarter page	...	**£3 0 0**

Series discount 10%
on four consecutive insertions

All communications respecting
Advertisements should be addressed to—

Mr. H. A. Collins
7 Lytton Gardens
Wallington, Surrey

Telephone: Wallington 1393

BRITISH JOURNAL OF CANCER

VOLUME XV, No. 2 JUNE, 1961

CONTENTS

PAGE

MILLAR, IAN B.—Gastro-intestinal Cancer and Geochemistry in North Montgomeryshire 175

CORNES, J. S., JONES, T. G. AND FISHER, GLORIA B.—The Incidence of Carcinoma in Patients Dying from Leukaemia, Malignant Disorders of Plasma Cells, and Malignant Lymphoma 200

KREYBERG, LEIV.—Main Histological Types of Primary Epithelial Lung Tumours 206

KREYBERG, LEIV AND SAXÉN, ERKKI.—A Comparison of Lung Tumour Types in Finland and Norway 211

CARROLL, R.—Changes in the Bronchial Epithelium in Primary Lung Cancer . 215

SALM, R.—Botryoid Sarcoma of the Vagina 220

COHEN, YAHYA AND HOE, SYLVIA.—Cancer of the Oesophagus in Singapore . 226

FRASER, L. E., SPICER, C. C., WILLIAMS, P. C. AND YOUNG, STRETTON.—Detection and Attempted Assay of Mammotrophic Activity in Women's Blood and Urine 243

BOYLAND, E., CHARLES, R. T. AND GOWING, N. F. C.—The Induction of Tumours in Mice by Intravaginal Application of Chemical Compounds 252

BIELSCHOWSKY, F. AND BIELSCHOWSKY, MARIANNE.—Carcinogenesis in the Pituitary Dwarf Mouse. The Response to Dimethylbenzanthracene Applied to the Skin 257

HOWELL, J. S.—Intranasal Administration of 9,10-dimethyl-1,2-Benzanthracene to Rats: the Development of Breast and Lung Tumours 263

BIANCIFIORI, C., BONSER, G. M. AND CASCHERA, F.—Ovarian and Mammary Tumours in Intact C3Hb Virgin Mice following a Limited Dose of Four Carcinogenic Chemicals 270

PERRY, D. J.—The Effect of Adrenalectomy on the Development of Tumours Induced by 2-acetylaminofluorene 284

ARCOS, J. C. AND GRIFFITH, G. W.—Ratio Dependent Synergism in Azo-dye Carcinogenesis 291

LAWS, J. O. AND YATES, SALLIE.—The Behaviour *in vitro* of Liver from Rats Treated with a Carcinogen 299

NAKAMURA, K. AND METCALF, D.—Quantitative Cytological Studies on Thymic Lymphoid Cells in Normal, Preleukaemic and Leukaemic Mice . . 306

METCALF, D. AND NAKAMURA, K.—Transplantation Bioassay of Thymuses from Preleukaemic AKR Mice for the Presence of Leukaemic Cells . . . 316

TOTH, B., DELLA PORTA, G. AND SHUBIK, P.—The Occurrence of Malignant Lymphomas in Urethan-treated Swiss Mice 322

CSABA, G., ÁCS, T., HORVÁTH, CECILIA AND MOLD, KATALIN.—Genesis and Function of Mast Cells. Mast Cell and Plasmacyte Reaction to Induced, Homologous and Heterologous Tumours 327

HARTVEIT, F.—The Survival Time of Mice with Ehrlich's Ascites Carcinoma related to the Sex and Weight of the Mouse, and the Blood Content of the Tumour 336

WILLIAMS, ANNA MARIA AND JORANGER, ELAINE.—Development of Mouse Ascites Tumour Sublines Resistant to Triethylene Melamine 342

DRAYTON, H. A.—The Inactivation by Organic Solvents and Detergents of Partially Purified Rous I Virus Preparations 348

Continued on p iii.

PRINTED BY ADLARD AND SON, LIMITED, BARTHOLOMEW PRESS, DORKING, SURREY.